ON A METHOD FOR RAPID DIGESTION OF BIOLOGICAL MATERIAL FOR ANALYSIS

by

ADOLPH BOLLIGER

(From the Urological Research Laboratory, University of Sydney).

(Submitted for publication 23rd March, 1932.)

PRINCIPLE.

The material under investigation is digested with perchloric acid, nitric acid, and hydrogen peroxide. If a dry ash should be required, the perchloric acid is evaporated, and due to the decomposition at a low temperature of the ammonium perchlorate formed, a nitrogen-free ash is easily obtained.

TECHNIQUE.

Tissues are preferably cut into pieces less than half a centimetre in size. About 1 grm. of the dried material is mixed with 4 to 5 c.c. 60% perchloric acid in a large pyrex test tube (1″ x 6″) or a small kjeldahl flask. Over a free flame the mixture is then brought to boiling. As soon as perchloric acid fumes appear the heating is interrupted, and 1 c.c. of concentrated nitric acid is added carefully drop by drop. Nitrous fumes appear, and in some instances foaming will make it necessary to shake the mixture well in order to avoid loss. Occasionally the foaming may be so bad that it will be necessary to add a drop of caprylic alcohol. When the reaction subsides the mixture is again heated, and more nitric acid is added as before. During this stage the solution has a yellow-brown appearance, but on further heating it may turn dark. In this case more nitric acid has to be added, till on further heating the mixture retains a clear yellow colour. In order to obtain an almost colourless solution it is advisable to add about 1 c.c. of 30% hydrogen peroxide drop by drop, followed by further heating and evaporation of part of the perchloric acid. If the solution is still not decolourized, this process has to be repeated till at least a light greenish yellow has been obtained. Undigested particles which may be still adherent to the walls of the vessel are dissolved by the perchloric acid fumes. On cooling, or after evaporating most of the perchloric acid, crystals appear, which in the case of material rich in protein consist mostly of ammonium perchlorate. Due to the solubility of the perchlorates formed, the

digest dissolves readily in water, with the exception of the potassium perchlorate, which requires for complete solution about 100 times its weight of water. Silica, which is occasionally present in physiological material, settles out as a white precipitate.

If a dry ash is required one takes either an aliquot part of wet digested material or less than 1 grm. of dried material if much protein is present. After the concentration of the wet digested material, or after the digestion of the substance, the remaining perchloric acid is evaporated to dryness. As soon as almost all of the perchloric acid has been evaporated the ammonium perchlorate decomposes instantaneously. After some further moderate heating a white ash results, if no large amounts of iron are present. By adding about another $\frac{1}{2}$ c.c. of perchloric acid and boiling for a few seconds all the cations present are converted into perchlorates. Last traces of ammonium perchlorate and organic material, which still may be present in a large sample, are decomposed by evaporating again to dryness.

Wet tissue requires less perchloric acid, and is digested in shorter time. One grm. of wet kidney tissue, for example, requires only from 2–3 c.c. of perchloric acid, and correspondingly less of nitric acid and hydrogen peroxide. Five grms. of dried faeces can be digested satisfactorily with 20 c.c. of perchloric acid, 15 c.c. of nitric acid, and 5 c.c. hydrogen peroxide. Sufficient perchloric acid should be taken to cover well the material to be digested, or an explosion may occur while heating over the flame.

If it is desirable, combustion can be effected by an amount of perchloric acid twice the weight of the dried material. Heat at water-bath temperature till the greater part of the material is liquefied, and then add concentrated nitric acid slowly in a quantity from two to four times the volume of the perchloric acid added. The reaction which takes place can be well controlled. If no further vigorous oxidation takes place after adding nitric acid at water-bath temperature, the digestion is completed over the flame.

In digesting easily digestible material, such as spinal fluid, blood filtrates or normal urines which contain only a moderate amount of organic matter, the use of a small amount of perchloric acid alone gives satisfactory results; 1 c.c. perchloric acid is sufficient for a speedy and satisfactory ashing of 10 c.c. of normal urine. The addition of nitric acid or hydrogen peroxide accelerates the process. One c.c. of 60% perchloric acid is sufficient for 1 c.c. of serum. Whole blood requires about 1·5 c.c. of perchloric acid per c.c., and the addition of nitric acid or hydrogen peroxide is necessary. In the case of very stubborn material it is advisable to alternate the addition of nitric acid with the addition of hydrogen peroxide.

The time required for the digestion of 1 c.c. of whole blood varies from 5–10 minutes, depending on whether or not dry ash is required; 1 grm. of wet liver or similar tissue takes about 10 minutes; 1 grm. of dried tissue or dried

faeces from about 10–20 minutes. Smaller amounts are digested in shorter time.

DISCUSSION.

In our numerous digestions we found hot perchloric acid by itself to be a powerful oxidiser of organic material. Violent reactions do not occur if sufficient perchloric acid is present in relation to the amount of organic material to be digested. Further safeguards are the addition of nitric acid or hydrogen peroxide, which allows the use of a considerably smaller volume of the less volatile perchloric acid. Together with hot perchloric acid, these substances represent a harmless and rapid oxidising mixture, which produces satisfactory results more quickly than any other process known to us. In general we prefer the addition of nitric acid, on account of its occasional greater efficiency, its known chemical purity, and its cheapness. But for the final destruction of certain residual yellow-coloured compounds, hydrogen peroxide was found to be more effective. If a nitrogen determination should be required, one would preferably use hydrogen peroxide alone.

Compared with the much practised digestion with sulphuric acid, perchloric acid has the advantage of greater oxidative activity at a considerably lower temperature. In 72% strength, it constitutes a "constant boiling point" mixture, boiling at 203°. Consequently any excess of perchloric acid is easily removed by evaporation. Furthermore, the presence of sulphuric acid interferes with many analytical procedures. On the other hand, the final digestion with perchloric acid is not always quite as complete as with sulphuric acid, but in most instances the organic compounds possibly remaining do not cause interference because they are completely soluble in water. If necessary they can easily be removed by adding sulphuric acid to the digestion mixture. The effectiveness of a sulphuric acid–perchloric acid mixture has been shown by Kennedy (1) and others.

By evaporating the perchloric acid to dryness an ash free from organic material and nitrogen is quickly obtained. The ammonium perchlorate, if present in large amounts, decomposes with a yellow flame and a hissing sound as soon as the mixture is almost dry. On account of this instantaneous decomposition only a fairly small amount of material can be converted into ash by this method, as otherwise the reaction may be too vigorous, and loss occurs.

Digestion with perchloric acid can be carried out in ordinary pyrex glass, as contrasted with ashing with alkalis, where special vessels are required. As compared with dry ashing, a method which has recently been criticized severely (2), the gain in time is very evident. In the case of smaller samples a perfectly white ash can be obtained within a few minutes, whereas it would usually take hours when subjected to dry ashing.

In general it may be said that most of the cations present as perchlorates

after digestion with perchloric acid lend themselves very conveniently to quantitative analysis. Further communications on the subject will deal with this aspect.

SUMMARY.

A simple method for the digestion and ashing of any type of biological material by means of perchloric acid, nitric acid, and hydrogen peroxide has been described.

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THE FILTRATION OF PROTEIN TUNGSTATE PRECIPITATES FROM UNLAKED BLOOD[1]

by

H. S. HALCRO WARDLAW

(From the Department of Physiology of the University of Sydney).

(Submitted for publication 23rd March, 1932.)

The method introduced by Folin (1930) for obtaining a protein-free fluid from unlaked blood involves spinning in a centrifuge within a short time after the protein has been precipitated. Filtration of these precipitates is not feasible, as the corpuscles soon begin to disintegrate. Usually their disintegration products begin to appear in the filtrate before filtration is complete, in sufficient quantity to render it turbid and coloured.

Spinning in a centrifuge is not always convenient when numbers of routine blood analyses are to be dealt with, but the difficulty in the way of filtration may be removed by the employment of an isotonic acid solution such as is used by Folin and Svedberg (1930) in one of their micro methods. The solutions used in the present work were as follows:

(1)	Sodium sulphate, anhydrous	15 grms.
	Sodium tungstate	6 grms.
	Water	1,000 c.c.
(2)	Sodium sulphate, anhydrous	15 grms.
	N/3 sulphuric acid	1,000 c.c.

The usual proportions of the reagents are employed: 1 volume of blood is mixed with 8 volumes of the tungstate solution. The plasma proteins are then precipitated by the careful addition of 1 volume of the isotonic acid solution, with gentle but thorough mixing. The material is then poured into a folded filter paper large enough to take it all and allow some margin. The precipitates obtained as described are very coarsely flocculent, and filter rapidly. The volumes of filtrate obtainable are relatively larger than those from Folin-Wu precipitates. Under the conditions observed 20 c.c. of the precipitation mixture ceased filtering in less than 15 minutes, and yielded an average of 13 c.c. of filtrate. An equal volume of the Folin-Wu mixture filtered much longer under

[1]The work described in this note was carried out in the Department of Biological Chemistry, Harvard Medical School, during the author's tenure of a Fellowship of the Rockefeller Foundation.

the same conditions, and rarely yielded as much as 10 c.c. of filtrate. Water clear filtrates were obtained through single thicknesses of Whatman No. 1 or Schleicher and Schüll No. 604 papers. These papers happened to be the first used. As they proved satisfactory others which may be equally suitable were not tried.

It is, of course, very important that specimens for analysis as unlaked blood shall be free from haemolysis when received. Much trouble was experienced in this respect with specimens obtained from routine collections in large hospitals. Any considerable amount of laking shows itself, owing to the fact that a clear filtrate cannot be obtained from the specimen. The presence of a smaller degree of laking is a more insidious danger. The proportions of the reagents used must be somewhat in excess of what is required for the precipitation of the plasma proteins alone. A perfectly clear filtrate will therefore be obtained even when small additional amounts of protein derived from the red cells are present. A clear filtrate is not evidence of complete absence of laking. It is impracticable to test every routine specimen for laking by spinning it down in a centrifuge. The measures necessary for the avoidance of laking are well known and easily applied. The occurrence of laking in some of the specimens examined in connection with the present work could be attributed to two causes:

(1) The use of needles which had not been properly freed from alcohol, and of wet syringes. The remedy for the first condition is obvious. It is not convenient, however, to wash and dry the syringe after each specimen when numbers must be collected, although this is the best procedure. Any danger of laking is avoided by using normal saline as a rinsing liquid instead of water.

(2) The use of excessive amounts of anticoagulants. A common practice is to collect the specimens in tubes containing an amount of powdered potassium oxalate in excess of that required to prevent clotting of a whole tubeful. When the tube is only partially filled, as usually happens, the excess becomes still greater. In some instances the condition is aggravated further by the addition of sodium fluoride to inhibit glycolysis. If the proportions of oxalate or fluoride are not allowed to exceed approximately 1 mg. per c.c. of blood, clotting is effectively prevented and laking and interferenc with precipitation are avoided. Folin's lithium oxalate cloth is equally effective and more convenient to use.

It should be noted that glycolysis proceeds rapidly in the isotonic sodium sulphate-tungstate solution. The proteins should therefore be precipitated and filtered off promptly after the dilution of the blood. For example, the concentrations of glucose in two specimens of blood, collected over oxalate but containing no fluoride, were 75 and 106 mgs. per 100 c.c. when estimated as soon as possible after dilution. The concentrations in duplicate samples allowed to stand for 4½ hours at 25°C. before precipitation were 60 and 82 mgs. per 100 c.c. respectively.

Barrenscheen and Hübner (1928) have shown that in the presence of the intact red cells the sulphate ion considerably accelerates glycolysis in blood.

The author wishes to express his indebtedness to Professor Otto Folin for the hospitality of his laboratory, in which this work was done.

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ON THE VIABILITY OF THE EGGS OF *SMINTHURUS VIRIDIS* L. (COLLEMBOLA) IN RELATION TO THEIR ENVIRONMENT

by

J. DAVIDSON

(Waite Agricultural Research Institute, University of Adelaide).

(Submitted for publication 8th April, 1932.)

I. INTRODUCTION.

A characteristic feature of the habits of *S. viridis* in South Australia is that during the dry season (November to April) the insect does not generally occur in an active form. Certain of the eggs remain viable in the soil during the summer and hatch at the beginning of the wet season, usually in April or early in May. During the winter period the insect is active in large numbers in certain areas of the State.

The two important factors responsible for this seasonal behaviour are soil moisture and temperature.

Owing to the high temperatures and extreme dryness of the surface soil during the dry season, the eggs are exposed to severe conditions. A study has been made, therefore, of the viability of the eggs of this species under various drought conditions.

In earlier papers soil factors affecting oviposition (1932) and the influence of temperature on the incubation period of the eggs (1931) in this species were discussed.

II. TECHNIQUE.

Surface soil taken from a Waite Institute paddock, to which 10–15% of sand was added, was used throughout the experiments. Its saturation value, calculated on moist soil, was 24% moisture. The soil moisture values given in the paper are calculated on the moist soil, and usually refer to the moisture content of the top ¼-inch of the soil.

Many difficulties were met with in connection with the maintenance of approximately constant moisture values in the surface soil during an experiment. In general, with ordinary flower pots, a fairly uniform moisture was maintained by keeping the pots in moist soil containing about 70% sand. In

experiments in which a particular moisture value in the soil was required for several days, various methods were tried. A method in which the weight of moisture lost daily was replaced by adding the required amount of water proved unsatisfactory. This was chiefly due to the uneven distribution of the water through the soil. The method finally adopted was to make up the soil to the required moisture value and reduce evaporation during the experiment as much as was practicable.

Oviposition cages similar to those already described (1932) were used. The eggs were laid on the exposed surface of the soil in cages in the open-air insectary. A Hearson seed-testing incubator was used.

As far as possible, average, well-formed egg batches, consisting of 30–50 eggs, were used in the tests. A rough estimate of the number of eggs, based on the size and formation of the batches, was made in most of the tests. These estimates, however, could only be used as a general guide to the number of eggs.

III. DESCRIPTION OF THE HATCHING OF THE EGGS OF *S. VIRIDIS*.

The egg is about 0·27 mm. in diameter. It is normally surrounded with a coating of soil-excreta. The chorion is relatively thick, and in older eggs its surface is somewhat roughened. Its inner surface is smooth, with a conspicuous silvery appearance after hatching.

Incubation is influenced by the temperature and moisture surrounding the egg. With eggs which have undergone considerable development, the pigmented eyes of the embryo are clearly visible through the chorion.

When the embryo is in an advanced stage of development, the egg swells and an equatorial slit develops in the chorion (fig. 1a). Gradually the slit becomes more pronounced, and the antennae are clearly visible through a delicate membrane which surrounds the embryo. The chorion becomes divided into two almost equal portions (fig. 1b), which gradually become widely separated, forming two polar caps. The antennae and legs, which are not yet fully developed, are clearly visible through the membrane investing the embryo (fig. 1c)[1]. Often both parts of the chorion are not completely separated.

Soon after this stage of development has been reached the movements of the embryo are plainly seen. By expansion and contraction of its body the head is freed from the anterior-cap-like portion of the chorion, the forelegs are extended, and the whole body is withdrawn from the chorion.

The emergence of the young insect from the egg is facilitated when the conditions are moist, and within a few minutes from the time it begins to push out of the enveloping membrane it is freely walking about. If the soil-excreta surrounding the eggs is dry and hard the insect may be unable to emerge.

[1] Ripper (1930) describes the changes which occur during the development of the egg of *Hypogastrura* which apparently differ in details from those observed in *S. viridis*.

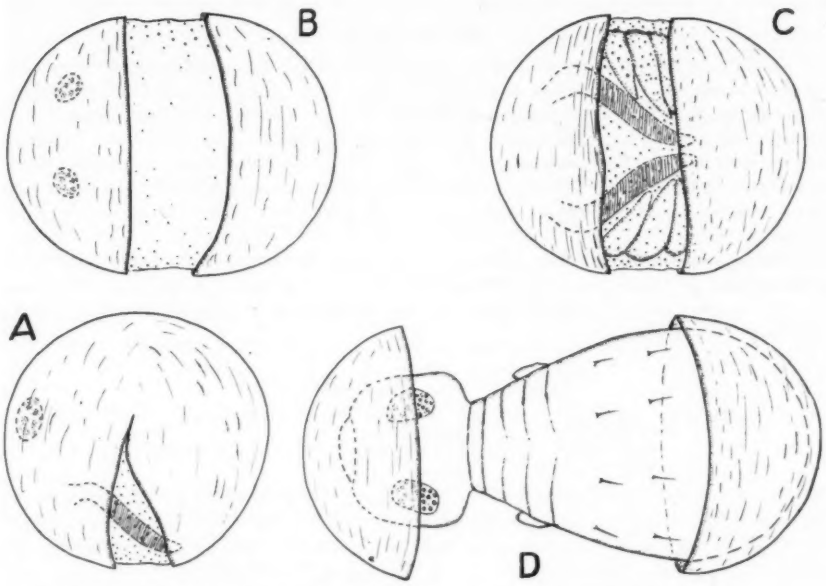

Figure 1. Four advanced stages in the development of the egg of *S. viridis*.

The newly-emerged insect remains for a few minutes to allow the cuticle to harden, with the antennae actively vibrating. I have observed the ventral tube used as a cleaning organ during this period. The delicate tubes were extended over the body several times and withdrawn across the mouth.

IV. ON THE HATCHING OF THE EGGS OF *S. VIRIDIS* IN RELATION TO SOIL MOISTURE.

Soil moisture and temperature are the important factors affecting the hatching of the eggs. It is the actual moisture content of the soil and soil-excreta surrounding the eggs, and not the atmospheric humidity, which is important, except in so far as the latter greatly influences the loss of moisture from the soil.

If low soil moisture values are maintained there will be a delay in the incubation period and a decrease in the number of eggs which hatch. Below a certain soil moisture value hatching will cease, but this value will differ according to the type of soil.[2]

[2] When the soil is drying out, the moisture content of the matrix in which the eggs are embedded, forming the egg batch, for a time, is slightly higher than the surrounding surface soil.

Experiment 1: Hatching data from egg batches incubated at 24°C, on soil having different moisture values.

Eight enamelled pots were used, each pot containing 6 kgs. of soil made up to the required moisture values. Five egg batches, laid within 48 hours, were lightly embedded over the surface of the soil in each pot. Each batch was enclosed with a 60-mesh wire gauze cylinder, ½ in. diameter and 1⅓ in. long. The remaining area of the soil surface was covered, one inch deep, with a gravel mulch. The open end of each gauze cylinder projected above the mulch and was closed with a glass square. Each pot complete was weighed and suspended in a water bath (Wisconsin tank) at a controlled temperature, which gave a temperature of 24 to 25°C on the surface of the soil.

The pots were weighed daily, and water added below the surface of the soil, to replace the moisture lost. The daily loss in weight varied from 6 to 10 grms. per pot.

The moisture content of the surface soil was calculated at the beginning and end of the experiment (Table 1).

TABLE 1. (See Experiment 1.)

Pot.	Soil Moisture (per cent.). Beginning.	End.	Hatching Commenced (days).	Remarks.
1	14	10	8	Large numbers of eggs hatched from each batch.
2	14	10	8–9	Large numbers of eggs hatched from each batch.
3	10	8	9	A number of eggs hatched from 4 batches. One batch, no hatch.
4	9	8	9–10	A number of eggs hatched from each batch.
5	8	7	10	Several eggs hatched from each batch.
6	7	7	10	Only 5 eggs hatched. From 3 batches no hatch.
7	6	3	—	No hatch.
8	4	3	—	No hatch.

Experiment 2: Hatching data from egg batches incubated at about 12°C on soil having different moisture values.

Four tins (9 in. x 6 in. x 2½ in. deep) each containing 1 kg. of soil made up to a different moisture content were arranged as in Experiment 1. On July 4th, 10 egg batches were placed on the surface of the soil in each tin. The tins were then placed in a closed box, embedded in moist sand, and kept in an outside laboratory. Under these conditions there was practically no loss of moisture from the soil. The mean temperature was about 12°C.

The eggs commenced to hatch as shown in Table 2, and the approximate number of eggs which hatched in each series, during the succeeding ten days, shows the influence of the soil moisture.

TABLE 2. (See Experiment 2.)

Tin.	Soil Moisture (per cent.). (Aug. 5th).	Date hatching commenced. (August).	Total hatch in 10 days (approx.).	Remarks.
1	14	2–3	540	From one batch, no hatch.
2	12	2–4	400	Hatching from all batches.
3	10	3–4	340	Hatching from all batches.
4	7	4–6	100	From four batches, no hatch.

With both experiments there was an increase in the incubation period in the lower soil moisture and a marked decrease in the number of eggs which hatched. In Expt. 1 the few which hatched at 7% moisture represent eggs favourably situated in the centre of the batches. In Expt. 2 the air in the box was almost saturated, which favoured hatching in tin No. 4.

V. ON THE VIABILITY OF EGGS OF *S. VIRIDIS* IN RELATION TO SOIL MOISTURE.

It has been shown that, with the particular soil used, there is a marked decrease in the number of eggs which hatch when the soil moisture value is below 7%. What happens to the eggs if the soil moisture is below this value for a long period? With high temperatures and low relative humidity they will be subject to severe desiccation. During the summer in South Australia the moisture content of the surface soil is well below this value for many weeks (*vide* Prescott and Piper, 1930), yet certain eggs remain viable.

A. HATCHING DATA FROM EGG BATCHES RETAINED ON MOIST SOIL—NORMAL HATCHING CURVES.

When the conditions for incubation are favourable, all the eggs in a normal batch do not hatch at the same time. A large percentage of the eggs hatch shortly after the first insects emerge, but a certain number exhibit delayed hatching. This appears to be due, apart from slight differences in age, to peculiarities in individual eggs, for instance, variable thickness of the chorion, and to the variable position of the eggs in a batch with reference to the surrounding temperature and moisture.

Experiment 3: Hatching data from normal egg batches.

(*a*) Five egg batches were incubated on moist soil at 25°C; hatching commenced on the 8th day; a total of 330 eggs hatched, of which 45% hatched on the 9th day.

(*b*) Ten egg batches were incubated at a variable temperature in the glasshouse; hatching commenced on the 22nd day; a total of 241 eggs hatched, of which 63% hatched on the 23rd day.

(*c*) Ten egg batches were incubated as in (*b*); hatching commenced on the 22nd day; a total of 290 eggs hatched, 72% of which hatched on the 23rd day.

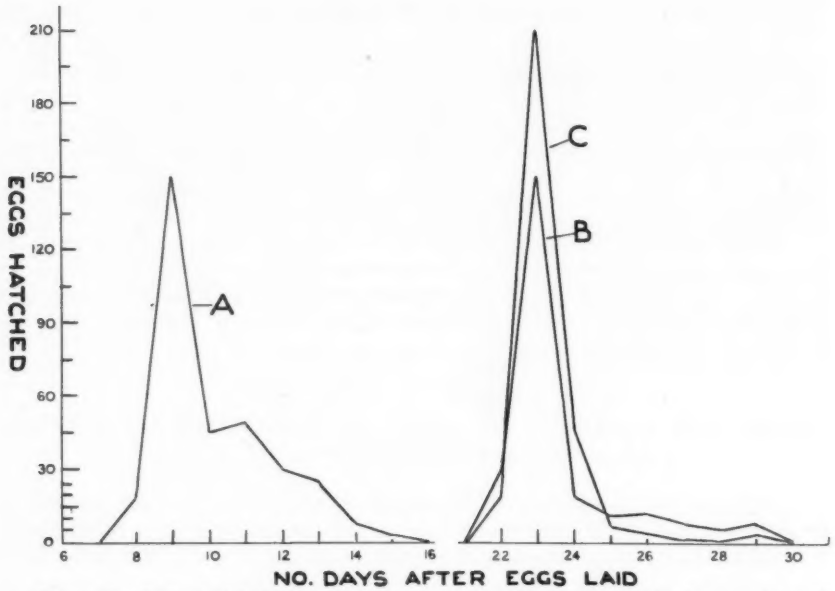

Figure 2. Daily hatch from egg batches of *S. viridis* when retained on moist soil. A at 25°C; B and C at a variable temperature in the glasshouse. (See Experiment 3.)

The hatching curves are shown in fig. 2. The batches in (a) were specially large ones, so that the total number of eggs was approximately the same as in (b) and (c), namely, 350 to 400 eggs.

B. Hatching Data from Egg Batches Removed from the Soil on which they are Laid and Dried for Various Periods.

When egg batches are removed from the soil within about 48 hours of being laid and exposed to dry conditions, a high percentage of the eggs may be killed. The number of survivors varies with different batches. This is partly due to variation in size and compactness of the batches in relation to the retention of moisture, and to the moisture content of the soil on which they were laid.

The egg has a minimum moisture requirement, below which it will not develop. If the available moisture is below this value the length of time the egg remains viable will depend upon (a) the severity of the dry conditions, (b) the resistance of individual eggs.

Experiment 4: Egg batches removed from the moist soil within 48 hours of being laid and dried in the laboratory from 6 to 12 days.

Fifty egg batches were removed from the soil and exposed in a tray in the laboratory, in June, for periods of 6 to 12 days, and then incubated on moist soil at 25°C.

With 46 batches, no eggs remained viable; from the remaining 4 batches 106 eggs hatched.

Experiment 5: Egg batches removed from the moist soil within 48 hours of being laid, and dried in the laboratory from 12 hours to 4 days.

Seventy-five batches were exposed in the laboratory as in Experiment 4, and then incubated on moist soil at 25°C (Table 3).

TABLE 3. (See Experiment 5.)

Series.	No. egg batches.	Days on moist soil.	Drying period (days).	Eggs hatched.	Day hatching commenced.
I	5	1	½	35	9
II	8	1	2	0	–
III	42	2	3	142	7
IV	20	2	4	205	5
(Controls)	5	Direct in incubator—not dried.		330	8

Experiment 6: Egg batches removed at periods of ½ to 4 days after laying, and dried in a heated glasshouse for 10 days, then incubated on moist soil (Table 4).

TABLE 4. (See Experiment 6.)

Series.	No. egg batches.	Days on moist soil.	Drying period (days).	Eggs hatched.	Day hatching commenced.
I	10	½	10	96	6
II	12	1	10	0	0
III	12	2	10	39	7
IV	10	3	10	19	5
V	10	4	10	124	5

Experiment 7: Egg batches retained on moist soil at 25°C for 2 to 6 days after being laid, then dried in a heated glasshouse for 8 days.

Five batches were placed in an incubator at 25°C (within 48 hours of being laid) on each of five successive days, commencing with July 24th. On July 30th the 25 batches were removed from incubator. On August 2nd they were placed in a heated glasshouse to hasten drying. At the end of eight days the batches were replaced on moist soil at 25°C, and the number of eggs which hatched daily in each series was recorded (Table 5).

TABLE 5. (See Experiment 7.)

Series.	No. egg batches.	Date eggs laid (July).	Days in incubator.	Eggs hatched.	Day hatching commenced.
A	5	23–24	6	17	2
B	5	24–25	5	58	2
C	5	25–26	4	47	2
D	5	26–27	3	94	3
E	5	27–28	2	106	4*

* Seven eggs hatched on 2nd day.

There is a decrease in the number of viable eggs with the increase in the incubation period given prior to the eggs being dried. This is due to the different stages of development of the embryos (see Expt. 10). Ripper (1930) refers to a resistant stage in the development of the egg of *Hypogastrura.*

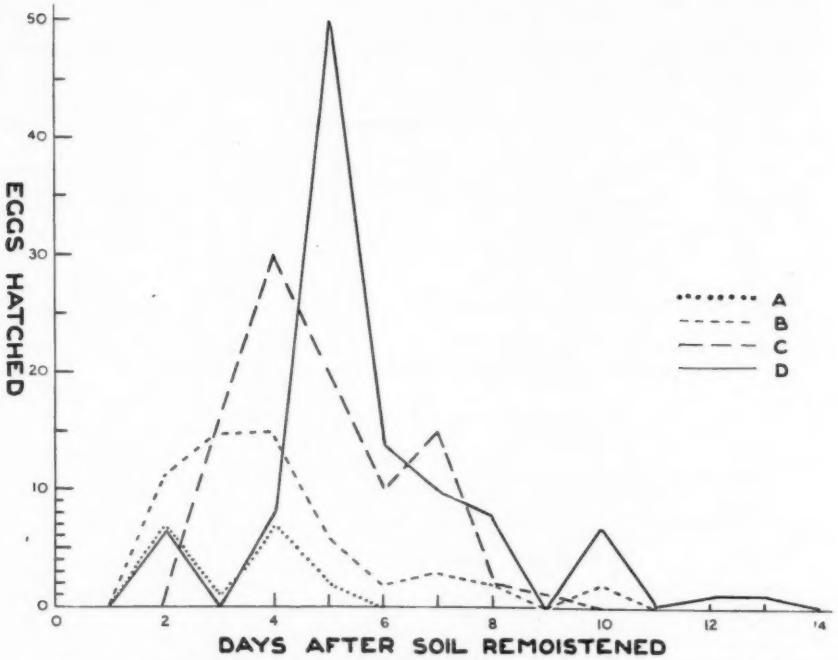

Figure 3. Daily hatch from egg batches of *S. viridis* which were incubated at 25°C for A, 6 days; B, 5 days; C, 3 days; and D, 2 days. They were then dried for 8 days, remoistened, and incubated at 25°C. (See Experiment 7.)

In series A to C many eggs would have completed more than 50% of their development when the batches were placed in the glasshouse. At this stage they were susceptible to the subsequent dry conditions. Many eggs contained dead embryos at the end of the experiment. In series D and E certain eggs would have made almost 50% development, since sufficient moisture would be available in the batches, for a time, after being placed in the glasshouse. At this stage they were more resistant to the dry conditions. The hatching curves are shown in fig. 3. Series C is not included.

In fig. 4A a combined hatching curve is shown. The points on the curve represent the total eggs which hatched daily from all the series in Expt. 7. A total of 322 eggs hatched, of which 49% hatched on the 4th and 5th days after the soil was remoistened.

The curve B is a theoretical curve. The points on it represent the daily hatching which would be expected from all the batches in Expt. 7 if the eggs had been retained in the incubator and not dried. They have been calculated

from curve A (fig. 2). The eggs would commence to hatch on the 8th day, and a total of 825 eggs might be expected to hatch.

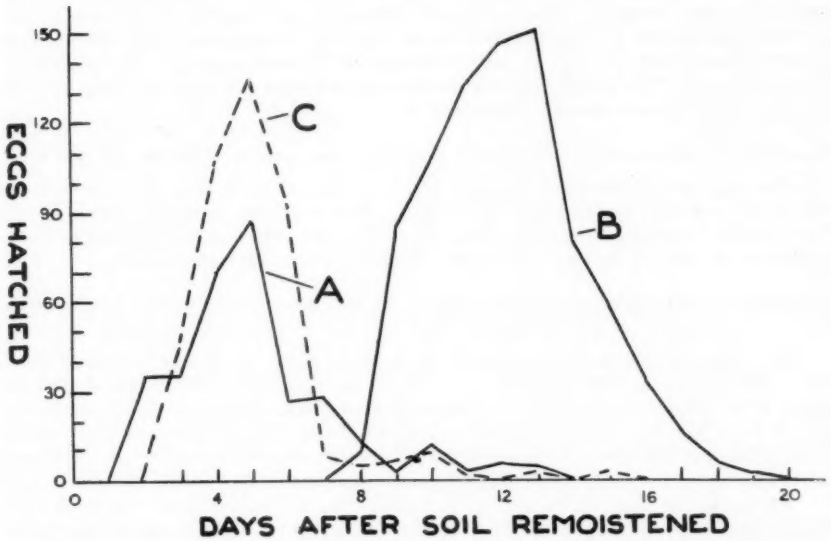

Figure 4. A: Total daily hatch from all the series in Experiment 7. B: Total daily hatch (calculated) which would be expected from all the series in Experiment 7 if the batches had been retained throughout on the moist soil at 25°C, and not dried. C: Daily hatch from egg batches which had 4 days' incubation at 25°C, then dried for 12 days, remoistened and incubated in the glasshouse. (See Experiment 8.)

The general shape of the curves A and B (fig. 4) is the same, but the greater mortality in Expt. 7 is reflected in the smaller size and greater irregularity of A.

C. Hatching Data from Egg Batches when the Soil on which the Eggs are Laid is allowed to Dry Out, the Batches being left Undisturbed.

The conditions of drying in the experiments in the previous section were artificial, and the loss of moisture from the batches more rapid and irregular than would occur normally in the field, where the egg batches remain undisturbed.

Experiment 8: Egg batches retained in situ on the soil, incubated for 4 days at 25°C and the soil then dried in a heated glasshouse for 12 days, mean daily Temp. 20°C.

Fifteen egg batches, laid on moist soil contained in 2½ in. flower pots on August 2–3, were incubated for four days at 25°C. On August 7th the pots were placed in a heated glasshouse to dry out for 12 days. On August 19th the moisture content of the soil was 1·2%. It was remoistened, and the pots were replaced in the incubator.

Hatching commenced on August 22nd (3rd day), and ceased on September 3rd. A total of 412 eggs hatched, of which 32% hatched on August 24th and 91% between August 22nd and 25th (Fig. 4C). The number of viable eggs is high, which is due partly to the non-removal of the batches from the soil, but chiefly owing to the fact that the majority of the eggs had reached a stage of development more resistant to the temporary dry conditions.

When egg batches are removed from the soil and dried shortly after being laid, they lose moisture rapidly, and few eggs are able to develop to the resistant stage. The mortality is therefore greater. If a certain degree of development has taken place before drying, the eggs are more resistant, as is shown in Experiment 9.

Experiment 9: Egg batches incubated for four days, then removed from the soil and dried.

Five egg batches, incubated on moist soil for 4 days at 25°C, were then removed and exposed in a tray in the laboratory for 3 days. They were then replaced on moist soil in the incubator. Hatching commenced 3 days later. A total of 134 eggs hatched, of which 45% hatched on the 4th day, and 45% on the 6th day (compare Experiments 5 and 6).

Experiment 10: Egg batches retained in situ on the soil, incubated for periods of 0 to 7 days at 24 to 25°C, and the soil then dried in a heated glasshouse for 17 days.

The eggs were laid on soil contained in 2½ in. flower pots. There were 8 series of 6 pots each. The eggs in the different series were laid on successive days. One series of pots was placed in the incubator each day, on 7 successive days, commencing August 8th, so that the various series had from 7 to 0 days incubation. On August 15th all the pots were removed to the glasshouse, and the soil allowed to dry out until September 1st (17 days). The mean daily temperature and relative humidity in the glasshouse during this period were: 21°C, 56%.

When removed from the incubator on August 15th, the soil moisture was about 16%. By the fourth day of the drying period (August 19th), it was 2%. The amount of soil in each pot was the same, and they dried out fairly uniformly.

On September 1st all the pots were remoistened and retained in the glasshouse, the number of eggs hatching being recorded daily (Table 6). Mean daily temperature was 19°C.

TABLE 6. (See Experiment 10.)

Series (six-pots).	No. egg batches.	Days in incubator 24–25°C.	Hatching commenced (September).	Eggs Hatched.
A	18	7	5	78 (a)
B	13	6	5	178 (b)
C	14	5	6	504
D	13	4	5	584
E	14	3	5	407
F	9	2	6	234
G	8	1	11	137
H	11	0	12	18

(a) Ninety-three eggs hatched during August 17th to 20th. No more eggs hatched until after the soil was remoistened, as its moisture was too low. These hatchings must be regarded as mortalities, since the newly hatched young died owing to the dry conditions. They are not included in this column.

(b) Eighty-six eggs hatched during August 18th to 20th. See notes under (a).

The hatching curves are shown in figs. 5 and 6. The largest number of viable eggs occurred in series D, E, and C. The egg batches in these series had 4, 3, and 5 days' incubation at 24°C. before being dried.

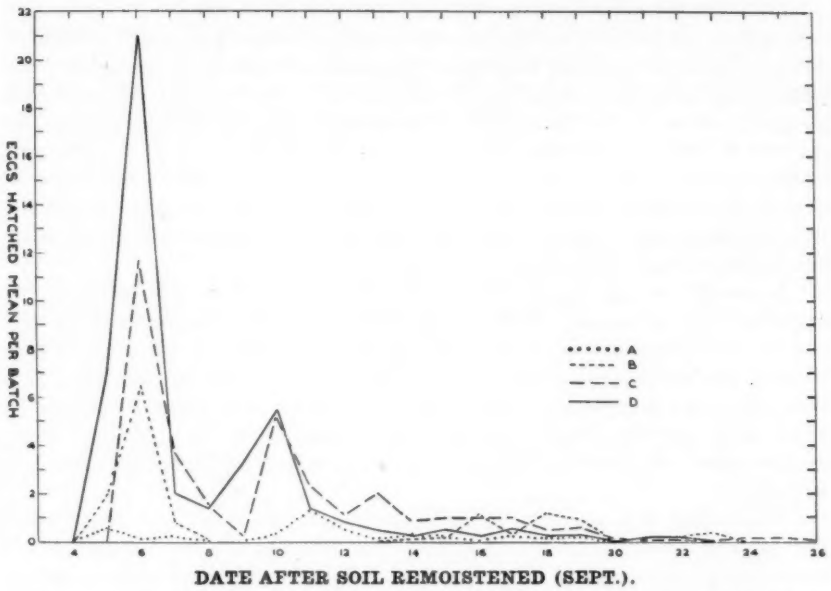

Figure 5. Daily hatch from egg batches of *S. viridis* which were incubated at 24°C for A, 7 days; B, 6 days; C, 5 days; D, 4 days; E, 3 days; F, 2 days; G, 1 day; H, 0 days. They were then dried for 17 days in a heated glasshouse, and afterwards remoistened. (See Experiment 10.)

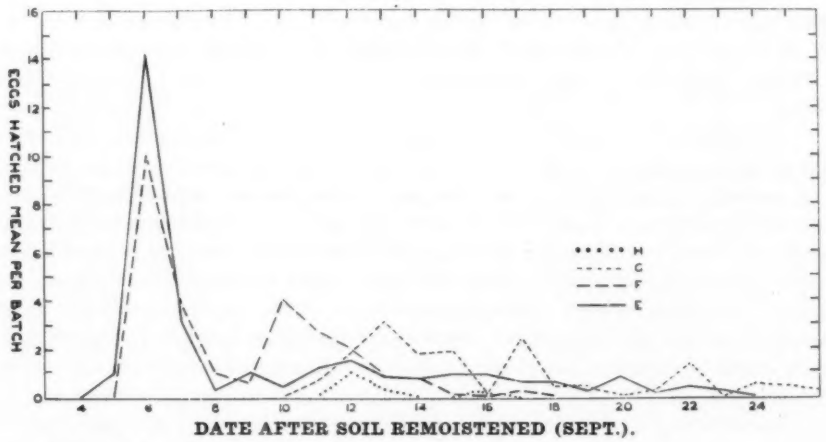

Figure 6. Legend as in Fig. 5.

With the remaining series there is a marked reduction in the number of viable eggs. In series A and B the majority of the eggs would have completed at least 75% of their development, when removed from the incubator on August 15th, since they were incubated for 7 and 6 days respectively. In fact, 93 eggs in series A and 86 in series B hatched during the first three days the soil was drying out. Further hatching was inhibited owing to the dry condition of the soil. It is evident that eggs which have undergone this advanced stage of development before the dry conditions set in are highly susceptible to the conditions, and a heavy mortality occurs. Dead embryos were found in many of these eggs at the end of the experiment.

In series C no eggs hatched during the drying period, as the embryos were not sufficiently advanced. Some of the eggs must have completed more than 50% of their development, since they had been incubated for 5 days. When the soil was remoistened, however, no eggs hatched until the 5th day. The steep rise at the beginning of the curves in this series, and also series B and D, shows that eggs which had undergone more than about 50% of their development when the dry conditions set in were killed by the subsequent dry conditions.

With series D it would be expected that many eggs had undergone about 50% of their development by August 15. With series E a less number would have reached this stage of development. Development of the eggs would not cease immediately the pots were removed from the incubator on August 15th.

Owing to specially favourable positions in the batches, certain eggs would have the moisture necessary for development for a short time after the commencement of the drying period (August 15th).

With series E to H the chances of the eggs being able to undergo 50% of their development, becomes less as the number of days' incubation is reduced. With series G and H the eggs did not commence to hatch until the 11th and 12th day, and a very small percentage of the eggs in these series remained viable.

The stage of development of the eggs in these experiments was not determined by examination of sectioned material. It is considered that the incubation period of dried eggs, when compared with that of normal eggs at the same temperature, will indicate whether any previous development has taken place. For instance, eggs incubated at 25°C. normally will commence to hatch on the 7th or 8th day, with a peak emergence the following day. If from egg batches which have been subjected to dry conditions the eggs commence to hatch on the 4th day after being remoistened, the short incubation period may be interpreted as due to development having taken place before the eggs were remoistened.

An outstanding feature of the hatching curves is the high peak which occurs on September 6th (5th day). In series B to F the following percentages

of the total viable eggs in each series, hatched on that day: 47%, 32%, 47%, 49%, 39%. These values are remarkably uniform, nothwithstanding that the eggs in these series were laid on successive days, and had been incubated before drying, for different periods. They show that at this stage of development the egg acquires a special resistance to drought conditions.

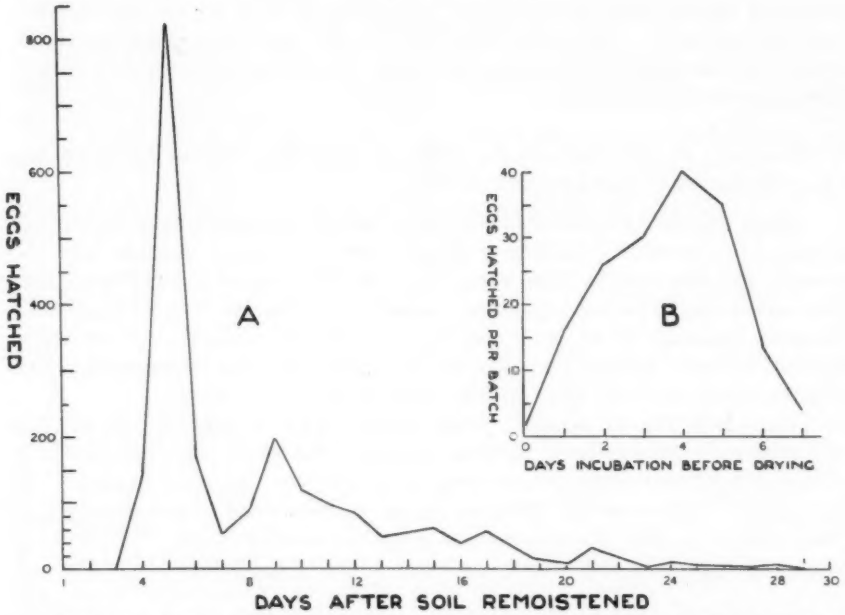

Figure 7. A: Total daily hatch from all the series in Experiment 10 (Figs. 5 and 6). B: The mean number of eggs per batch which hatched in each series of Experiment 10 is plotted against the number of days incubation prior to being dried.

The remaining viable eggs in these series hatched on various days up to September 29th. Even with series A and B a few eggs hatched up to September 23rd. Considering that these eggs had 7 and 6 days' incubation at 24°C. before being dried, the delay in hatching is remarkable.

Delayed hatching with certain eggs occurs with normal egg batches (*vide* fig. 2), but is more pronounced in batches which have been subjected to a temporary drought period. It appears to be due to variation in size and compactness of the egg batches affecting the relative protection afforded to particular eggs and to the individual resistance of particular eggs, for instance, thickness and permeability of the chorion. These eggs may be an important factor in enabling the species to withstand severe drought conditions.

In fig. 7A a combined hatching curve is given. The points on the curve represent the total number of eggs which hatched daily from all the series (100 batches) in Expt. 10. A total of 2,144 eggs hatched. Of these 38% hatched on September 6th (5th day). The steepness of the curve shows the resistance of eggs which had reached that particular stage of development.

In fig. 7B the mean number of eggs per batch which hatched in each series is plotted against the number of days' incubation at 24°C. which they received before being dried. The curve illustrates clearly that the greatest resistance to the dry conditions occurred amongst eggs which received from 3 to 5 days' incubation before being dried.

D. Viability of the Eggs of S. viridis in Relation to the Severity and Duration of the Drought Period.

Apart from the resistance of particular eggs to dry conditions, the number of eggs which survive a temporary drought period in nature depends upon the severity and duration of those conditions. In this respect the following considerations are important: (a) The character of the soil and its vegetative covering, especially in relation to its moisture-holding capacity. (b) Meteorological features influencing evaporation, especially the temperature and relative humidity of the air. (c) The duration of the dry period.

There is a definite moisture value, below which development of the egg will cease. If the available moisture remains below this value the egg may remain in a viable resting state, so long as it retains the moisture necessary for its vitality. Such essential moisture, in the extreme condition, is retained as "bound water." The temperature and relative humidity of the air during the drought period is the most important factor affecting the desiccation of the egg.

Experiment 11: Egg batches laid on moist soil and the latter allowed to dry out for 10 days in the open-air insectary.

(*a*) A number of egg batches were laid, during June 7th to 16th, on moist soil in tins as used in Experiment 2. The tins were then left exposed in the open-air insectary until June 26th. On that date 10 egg batches were removed and incubated on moist soil at a variable temperature in the glasshouse.

The eggs commenced to hatch on July 11th (15th day), and ceased on July 26th. A total of 221 eggs hatched, of which 28%, 15%, and 23% hatched on the successive days July 14th, 15th, and 16th. From 3 batches no eggs hatched. The hatching curve was irregular, owing to the death of many eggs, and the extended oviposition period.

(*b*) A number of egg batches were laid on moist soil contained in 4 in. flower pots during June 7th to 16th, and exposed in the open-air insectary until June 26th. On that date 20 batches were removed and incubated as in (*a*).

Hatching commenced on July 10th (14th day), and a total of 389 eggs hatched.

(*c*) Egg batches laid on June 26th, and incubated directly on moist soil in the glasshouse, commenced to hatch on July 20th (25th day).

The number of eggs in (*a*) and (*b*) which survived the dry period was comparatively high (compare Experiments 4–6). The winter conditions in the open-air insectary were not severe, the mean daily temperature and relative humidity during June 16th to 26th being 12°C and 79%.

Certain of the eggs evidently had undergone some development prior to the soil being remoistened on June 26th, since they commenced to hatch 10 days earlier than in (*c*).

Experiment 12: Egg batches laid on moist soil and the soil then dried for periods of 10 to 40 days in a heated glasshouse.

Egg batches were laid on moist soil contained in 25 flower pots (2½ in. diameter) during August 27th to 29th. Each pot contained an equal weight of soil, and a uniform soil moisture was maintained during the oviposition period.

On August 29th all the pots were placed in a heated glasshouse, and the soil allowed to dry. By August 31st the moisture content of the soil was 5%, and 2 days later 2·5%.

Five pots were remoistened every ten days, commencing with September 8th, and retained in the glasshouse, the soil being kept moist. The hatching data are given in Table 7.

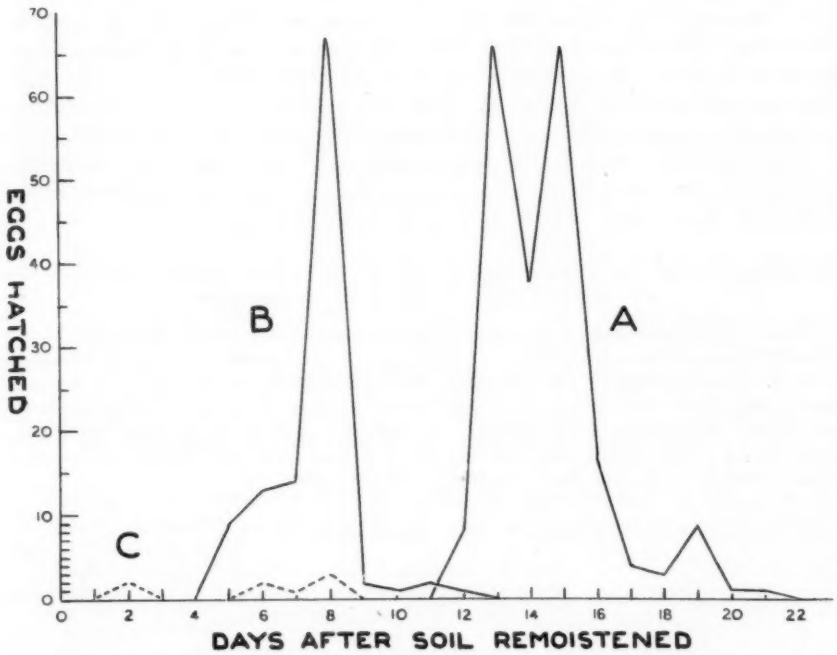

Figure 8. Daily hatch from egg batches of *S. viridis* which were dried for A, 10 days; B, 20 days; C, 30 days, in a heated glasshouse, then remoistened. (See Experiment 12.)

TABLE 7. (See Experiment 12.)

Series (5 pots).	No. egg batches.	Drying period (days).	Mean Daily. Max. T.	Min. T.	R.H.	Day hatching commenced.	Total eggs hatched.
A	8	10	90°F	52°F	55%	11	213
B	8	20	88°F	52°F	58%	5	109
C	8	30	91°F	52°F	55%	2	8
D	8	40	—	—	—	—	0
E	8	50	—	—	—	—	0

The hatching curves are shown in fig. 8. The decrease in the number of viable eggs with the extension of the dry period is very pronounced. These eggs were laid on the surface of the soil, and were freely exposed to the dry conditions. It is remarkable that some eggs were able to make considerable development during that period, notwithstanding that the moisture value of the surface soil was below 2·5% practically the whole period.

With series (C) only 8 eggs survived. Two of these hatched on the 2nd day after the soil was remoistened, and the remaining 6 on the 6th to the 8th day. Similarly with series (B) the eggs commenced to hatch on the 5th day. The normal incubation period for untreated eggs at these temperatures would be about 15 days. Hence in series (B) and (C) many eggs had undergone considerable development before the soil was remoistened. The explanation appears to be that the moisture in the excreted material surrounding the eggs is retained longer than in the surrounding soil. The degree to which this moisture is abstracted from the egg batch depends upon the formation of the latter and the severity and duration of the drought conditions.

Experiment 13: Egg batches laid on soils having different moisture content, and the soil then allowed to dry out for 14 days in a heated glasshouse.

The moisture available for the eggs during a temporary dry period will depend on the moisture content of the soil when the dry period sets in. In Experiment 13, 4 series of batches were laid on soil made up to 17%, 15%, 14%, and 10% moisture content, contained in tins as used in Experiment 2. The tins were placed in the heated glasshouse with the surface of the soil exposed for 14 days. The soil moisture values were calculated at intervals. At the end of the drying period the egg batches were removed to moist soil, and incubated in the glasshouse (Table 8). The mean daily temperature was about 20°C.

TABLE 8. (See Experiment 13.)

Series.	No. egg batches.	Soil moisture during drying period (%). 1st day.	7th day.	14th day.	Day hatching Commenced.	Ceased.	Total eggs hatched.
A	14	15	3·5	1·5	3	10	408
B	5	14	2	1·2	4	13	110
		1st	5th	14th			
C	18	12	9	1·5	5	13	640
D	4	8·5	1·2	1·2	9	13	28

Note. Series A and B were started a week later than C and D. The hatching curves for each series are given in Fig. 9.

With series A a normal type of curve was obtained (*vide* fig. 2). The batches were well formed. With series B and C the hatching curves are more irregular, due to the variable development of the eggs.

In series D few eggs remained viable. The batches were loosely formed, owing to the low moisture content of the soil during oviposition, and compared with Series A the eggs were exposed for a longer period to harmful low moisture conditions.

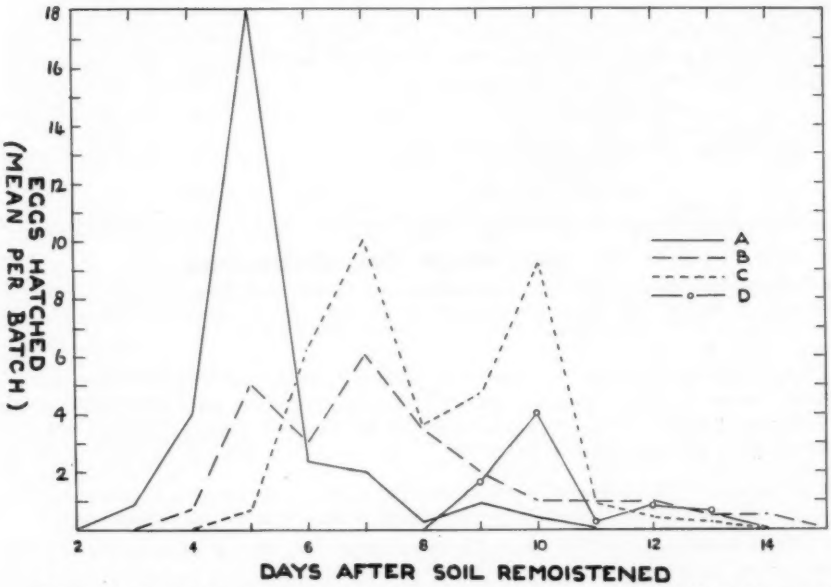

Figure 9. Daily hatch (mean per batch) from egg batches of *S. viridis* laid on soil having A, 17% moisture; B, 15%; C, 14%; D, 10%. The soil was allowed to dry out in a heated glasshouse for 14 days, then remoistened. (See Experiment 13.)

In this experiment the drying conditions were severe. In Expt. 14 the batches were dried under open-air conditions, and the mortality was relatively small.

Experiment 14: Egg batches laid on soil having a moisture content about 12%, the soil then being allowed to dry out for 16 days under out-door conditions.

A number of egg batches were laid during July 15th to 21st on soil having about 12% moisture content. The soil (4 kgrs.) was contained in a glazed earthenware pot, and the surface, with the eggs *in situ*, was exposed in the open-air insectary until August 7th. The mean daily temperature and relative humidity during this period was 51°F and 77%. On August 7th the moisture content of the surface soil was 2·5%.

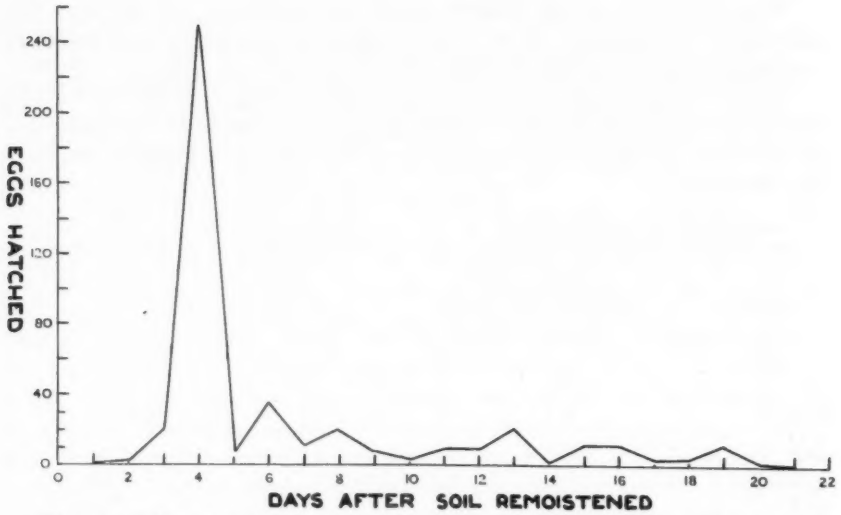

Figure 10. Daily hatch from egg batches of *S. viridis* laid on soil having about 12% moisture. The soil was allowed to dry out in the open-air insectary for 16 days, then re-moistened, and the eggs incubated at 24°C. (See Experiment 14.)

On August 7th the eggs were removed to moist soil and incubated at 24°C. The hatching curve obtained is shown in Fig. 10. A total of 449 eggs hatched, of which 55% hatched on the fourth day (August 11th). Hatching ceased on the twentieth day. A comparatively high percentage of the eggs remained viable.[3]

Experiment 15: Egg batches laid during a period of ten days on soil having a variable moisture content, the soil then allowed to dry out for 50 days under out-door conditions.

The aim in this experiment was to reproduce somewhat the conditions obtaining in the field during a rainless period at the end of the winter.

(*a*) Plots of clover seedlings were established in 29 tins, as used in Experiment 2. Each tin was infected with *S viridis* on September 8th, and no water was added to the soil after September 12th. The insects were removed on September 19th, the tins being left in the open-air insectary, with the soil surface fully exposed to the sun. A number of egg batches were laid. The moisture content of the surface soil on September 20th was 6%; on October 28th it was 1·2%.

On October 17th, 15 batches were removed and incubated on moist soil at 25°C. Two eggs hatched on October 21st, one on October 24th. No further eggs had hatched by November 2nd.

Owing to the soil drying out during the oviposition period, cracks appeared in the surface, and eggs were laid in these situations. The eggs removed on October 17th were taken from the exposed surface of the soil, where the drying conditions were severe. Those laid in the cracks were better protected.

[3] Owing to the low moisture content of the soil during the oviposition period, some of the egg batches were badly formed, so that a reliable estimate could not be made of the number of eggs used in the experiment.

On November 2nd all the tins were placed in a room having a variable temperature with a daily mean about 20°C, the soil being remoistened. The eggs commenced to hatch on November 5th, and during the succeeding 7 days the recorded number of eggs hatching daily was, 140+445+226+84+44+4+2=945. It was impossible to estimate the number of eggs laid, but considering the conditions during the drying period, a fair number of eggs remained viable.[4] The short incubation period shows that many of the eggs had undergone considerable development.

(*b*) A larger plot of clover seedlings growing on soil 4 in. deep, in a zinc tray 3 ft. x 2 ft., was treated as in (*a*).

On October 26th 6 egg batches were removed and incubated on moist soil at 25°C. From one batch 22 eggs hatched on October 31st. No further eggs hatched by November 15th. On November 15th 17 egg batches were removed and incubated at 25°C. Twenty eggs hatched from 2 of these batches on November 19th, and 17 on November 20th. From another batch one egg hatched on November 20th. No further eggs hatched from any of the batches by November 23rd. The viable eggs had developed considerably before being remoistened.

VI. ON THE VIABILITY OF THE OVER-SUMMERING EGGS OF *S. VIRIDIS* IN SOUTH AUSTRALIA.

The climatic conditions in the Adelaide area of South Australia are characterized by winter rainfall and summer drought. The essential meteorological features of the period November to April are given below, the figures being taken from the Official Year Book of the Commonwealth of Australia, No. 24 (1931).

Mean Monthly.	Nov.	Dec.	Jan.	Feb.	March.	April.
Rainfall (inches)	1·14	1·02	0·71	0·73	1·01	1·71
Number of days rain	7	6	4	4	6	9
Temperature, Fahrenheit	67	71·1	73·8	74·1	69·8	64
Relative Humidity (%, 9 a.m.)	42	39	38	40	46	55
Evaporation (inches)	6·62	8·45	9·01	7·36	5·88	3·52

The mean annual rainfall is 21 inches, of which only 28% falls during the period November to April. The mean annual evaporation is 55 inches, of which 74% occurs during this same period.

The average monthly temperatures of the soil, to a depth of 1 inch, at the Waite Institute during November to April, based on records for the five years 1925-29, were 25·1, 28·0, 28·9, 27·9, 25·8, 19·9 degrees C.

Under the conditions outlined above, the surface soil is very dry during the dry season (*vide* Prescott and Piper, 1930.)

A. The Proportion of Viable Eggs which Survive the Dry Season.

It is evident that the eggs of *S. viridis* are exposed to severe dry conditions during the dry season. Considering the results obtained in the experiments in the previous sections, it would be expected that comparatively few of the eggs would remain viable throughout the season.

[4] The mean daily temperatures and relative humidity during September and October were: mean maximum, 67°F., 73°F; mean minimum, 49°F., 55°F; relative humidity, 52%, 52%.

Experiment 16: The number of viable eggs in the soil, of infested areas, on various dates during the dry season.

Four square feet of surface soil and vegetable debris, to a depth of about half an inch, were collected on various dates from a Waite Institute paddock, where the insect is active in large numbers during the winter season. It was watered and kept in tins, as used in Experiment 2, in the laboratory, the number of individuals which hatched being recorded.

(*a*) Soil Collected November 3rd, 1931.

A few living individuals almost adult, were present in the debris when collected. Eggs commenced to hatch on November 5th. By November 7th, 274 eggs had hatched from the material.

(*b*) Soil Collected November 30th, 1931.

No living individuals were present. One egg hatched on the 6th, 9th, 10th, and 12th December, three on the 13th, and three on the 14th December.

(*c*) Soil Collected on December 14th, 1931.

No living individuals were present. One egg hatched on December 18th. No more eggs hatched by January 8th. Owing to a heat wave during this period, the high temperatures in the room were unfavourable.

(*d*) Soil Collected on February 22nd, 1932.

No living individuals present. Three eggs hatched on February 26th, and a few individuals on succeeding days. By March 14th a total of 27 eggs had hatched.

(*e*) Soil Collected on March 31st, 1932.

There were 10 wet days in March (1·637 inches), and the eggs commenced to hatch in the paddock at the beginning of April.

It is clear that there is a high mortality amongst the over-summering eggs. Owing to the varied local situations in the soil, under field conditions, in which eggs are laid, the conditions are less severe for certain eggs. The character of the dry season will also affect the number of viable eggs present in the soil at the beginning of the wet season, resulting in a heavier or lighter early infestation in the paddocks.

B. The Influence of Summer Rainfall on the Viability of Eggs in the Soil.

Owing to the high temperatures which prevail during the summer months, the low soil moisture actually favours the preservation of the species during that period. The resting condition of the over-summering egg is not a definite rhythmical diapause, but an enforced condition imposed by the absence of moisture necessary for development. The falls of rain in the summer months are normally too light and infrequent to maintain a favourable moist condition of the surface soil for more than a day or so. If a suitable soil moisture is maintained for a sufficiently long period, the eggs will develop and hatch, but the insects will not survive the subsequent hot, dry conditions.

This is well shown in the following experiment.

Experiment 17: Effect of summer irrigation of lucerne plots infected with eggs of S. viridis.

Three plots of lucerne, 9 yards by 6 yards (var. Hunter River), sown in 1926, which are always infested with *S. viridis* during the wet season, were irrigated in January, 1931, for various periods.

Plot A was watered on alternate days from January 11th to 25th, the amount of water given being roughly equivalent to 3 in. of rain.

Plot B was watered daily from January 11th to February 9th with the equivalent of roughly 6 in. of rain.

Neighbouring control plots were not irrigated.

On February 12th feeding marks were observed on the plants in both plots, and several young insects were found, particularly on plot B. None was to be found on the neighbouring control plots. The lucerne was cut on February 16th, and the insects died out during the next few days.

On the non-irrigated plots the insects began to hatch out in the second week in April. By April 27th they were present in fair numbers. By May 30th the lucerne on these plots was severely damaged by the insects, the growth being checked.

On plot B only a few isolated individuals were present at the end of May, and certain of these were doubtless migrants from the neighbouring plots. The lucerne had made conspicuous growth, and only a few feeding marks of the insect were found on the leaves.

The irrigation of plots A and B in January had resulted in the eggs of *S. viridis* developing. Certain of them hatched, and the insects died out, owing to the subsequent dry conditions. With other eggs the embryos were killed. The result was less pronounced in plot A, which received less water than B. Frequent irrigations are necessary, owing to the high evaporation rate, in order to maintain suitable moisture in the surface soil.

The irrigation tests were repeated in 1932, but later in the season. Commencing February 25th, one plot of lucerne was watered daily for 25 days. Another plot was watered on alternate days during the same period, but received the same total amount of water. A number of the insects hatched out during the first week in March, and by the second week they were present in fair numbers.

Hatching did not take place in non-irrigated areas until early in April.

VII. GENERAL DISCUSSION.

The eggs of *Sminthurus viridis* are laid in batches consisting of a variable number of eggs. The batches are formed by a matrix consisting of material excreted by the female in which the eggs are embedded. Each egg therefore is normally surrounded with a relatively thick covering of the excreted material which safeguards it against rapid loss of moisture during temporary dry periods (*vide* Davidson, 1932).

During the later stages in the development of the egg an equatorial slit appears in the chorion, which becomes divided, partially or completely, to form two polar caps. The embryo is invested with a membranous envelope. If

the batches are dry and hard, the young insects may be unable to emerge, and die owing to the dry conditions.

The moisture content of the surface soil on which the batches are situated gives a good indication of the moisture in the soil-excreta surrounding the eggs, but the moisture in the latter material, when the soil is drying out, appears to be retained longer than in the surrounding surface soil.

With Waite Institute surface soil, few eggs hatched when the soil was maintained at a moisture content about 7% moisture. Below 5% moisture no eggs hatched.

It is difficult to consider independently the effect of hard, dry egg batches in preventing the actual hatching of the eggs, owing to the influence of the low moisture conditions on the vitality of the embryos.

With a soil that is drying out the eggs will continue developing so long as the necessary moisture is available. Certain eggs may continue development when the moisture content of the surrounding surface soil is below 5%, owing to their protected position in the egg batch. The incubation period will depend upon the moisture available and the temperature (Davidson, 1931). When the moisture surrounding the eggs falls below the value necessary for their development, the factor of lethal desiccation may be involved. The resistance of the eggs to subsequent dry conditions will largely depend upon the stage of development attained and the temperature and relative humidity of the air surrounding them.

The experimental results indicate that, under natural conditions on Waite Institute soil, development of the eggs is retarded when the soil moisture is maintained below about 10%, and hatching does not take place with soil moisture values maintained below about 5%. When the soil is drying out, certain eggs, favourably situated in a batch, may continue developing for a time after the surrounding surface soil has reached this low moisture value.

When egg batches, laid within a period of 24 to 48 hours, are maintained under favourable conditions, the majority of the eggs will hatch within about 24 hours from the time hatching commences. The remainder of the viable eggs will hatch during the next few days (fig. 2). The delayed hatching is due to (a) time the eggs were laid; (b) variation in formation and compactness of the batches, which affects the position of certain eggs with regard to moisture and temperature; (c) variation in individual eggs, particularly as regards the thickness and permeability of the chorion.

If the egg batches are subjected to dry conditions certain eggs may be killed. The mortality depends chiefly upon the severity and duration of the conditions. The eggs are able to withstand a certain degree of dryness at all stages of development, but they are particularly susceptible to such conditions when the embryos are in an advanced stage of development. Eggs which have attained a stage of development obtained by 4 days' incubation at 25°C., when

the dry conditions set in, are more resistant to temporary drought periods (Expts. 8 and 10). This variation in resistance is evidently related to the changes which occur during the development of the embryo. Certain eggs exhibit a marked delay in hatching (figs. 5, 6, 7A, and 10). The reason for this is not clear, but is evidently due to the individual resistance of particular eggs, and may be important in nature in carrying the species over extended drought periods.

When egg batches are removed from the soil within a day or two of being laid and allowed to dry for a few days, a large number of the eggs are killed (Expts. 4 and 5). If the batches are left undisturbed on the soil on which they are laid, and the soil allowed to dry out, a much larger proportion of the eggs remain viable.

In the former instances, owing to the exposure of the under-surface of the batches, there is a rapid loss in the moisture surrounding the eggs compared with the latter. In the latter instances the batches are in contact with moisture in the soil, for a time, after the latter commences to dry out. This allows certain of the eggs to undergo development, and some of them may attain a stage more resistant to the subsequent dry conditions. Owing to the variation in size, formation, and compactness of the batches, and the varied situations on the soil surface on which they are laid, the percentage of viable eggs in dried batches may vary considerably.

The number of eggs which survive a drought period is greatly affected by the severity and duration of the drought conditions. In this respect, the temperature and relative humidity of the air (Expts. 12 and 14), and the moisture content of the soil when the dry period sets in (Expt. 13) are important factors. During the winter months in South Australia a large proportion of the eggs will survive temporary dry periods.

Owing to the hot, dry conditions during the summer, comparatively few of the eggs present in the soil of infested areas, at the end of the wet season, survive the subsequent dry season (Expt. 16). Doubtless eggs which are freely exposed on the surface of the soil are killed (Expt. 12). However, many batches are laid in more favoured situations, in crevices in the soil or under surface debris, and certain of the eggs remain viable, the number depending upon the character of the dry season (Expt. 16).

The dormant condition of the over-summering egg is enforced by the absence of moisture necessary for its development. It is not a necessary rhythmical diapause, since hatching can be induced by giving suitable conditions of moisture and temperature (Expt. 17). During cool, wet periods some eggs may hatch in the field during the summer, but the conditions would be unfavourable for the multiplication of the insects, and normally they would not survive subsequent dry conditions.

VIII. SUMMARY.

1. *S. viridis* is active in large numbers in certain areas of South Australia during the wet season (May to October). Normally it does not occur during the dry season. Certain eggs remain viable in the soil during the latter season. The dormant condition is not a definite rhythmical diapause, but one enforced by the dry conditions, which arrest development of the egg.

2. Experiments have been made on the influence of dry conditions on the viability of the eggs of this species.

3. With Waite Institute surface soil, the development of the eggs is retarded when the soil moisture is maintained below about 10%. If maintained below about 5% moisture, they do not hatch.

4. When egg batches are removed from moist soil within a day or two of being laid, and allowed to dry, many eggs are killed. The mortality is not so great if the batches are left undisturbed on the soil and the latter allowed to dry out.

5. The number of eggs which survive the dry conditions depends chiefly on (a) the stage of development of the eggs when the dry conditions set in, (b) the severity and duration of the conditions, especially with reference to the temperature and relative humidity of the air.

6. Eggs which have attained a stage of development represented by about 4 days' incubation at 25°C. are more resistant to temporary dry conditions.

7. This resistance is related to the changes which occur during the development of the embryo.

8. Certain eggs in a batch exhibit delayed hatching. This appears to be due to (a) the position of the eggs in the batch, and the size and formation of the latter, (b) the individuality of particular eggs, probably in regard to the thickness and permeability of the chorion.

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THE PHYSIOLOGY OF XEROPHYTISM IN AUSTRALIAN PLANTS

THE CARBOHYDRATE METABOLISM OF PLANTS WITH TOMENTOSE SUCCULENT LEAVES.

by

J. G. WOOD

(Department of Botany, University of Adelaide, South Australia).

(Submitted for publication 22nd April, 1932.)

The so-called xerophytic plants of Australia fall into two groups, the sclerophyll-leaved plants and the tomentose succulent-leaved plants. In another place (1931) the writer has discussed the stomatal frequency, transpiration, and osmotic pressures of these two groups, and whilst differences have been observed they afford no basis for the determination of the cause of sclerophylly or of semi-succulence.

Investigations have shown that in the two types carbohydrate metabolism is directed into two channels. In the succulent-leaf type part of the carbohydrate flux is directed towards pentosan formation, whilst in sclerophyll leaves the formation of uronic acid complexes and of aromatic lignin compounds occurs. These metabolic changes give the particular character to the plants concerned.

CARBON ASSIMILATION.

The bearing of carbon assimilation on the problem of xerophytism has been neglected to a large degree, although this process, especially as controlled by temperature, is of the utmost importance in the economy of the plant. The limits of plant distribution are controlled by the temperature of freezing and the lethal temperature.

In the Koonamore district, which may be taken as a typical area of the shrub steppe, from December to March the mean shade temperature is about 72°F., and thermohygrograph records show that during these months the shade temperature on the average is above 90°F. (32°C.) between the hours of 9 a.m. and 9 p.m.

It is during these three months, following rains, that the maximal growth of *Atriplex vesicarium* occurs.

It is a fact of general comment that saltbushes make but little growth during mild summers, as, for example, that of 1929-1930.

It is expected, therefore, on *a priori* grounds, that the assimilation/temperature relations in these plants should differ considerably from those of more mesophytic plants.

The work of Lundegardh (1931) and others has shown that in mesophytic plants the optimum temperature for assimilation lies between 15° and 30°C. in full sunlight and 0·03% CO_2.

To establish the relations in shrub-steppe plants the character plant of the plains *(Atriplex vesicarium)* was used, and its assimilation measured under natural conditions.

The rate of assimilation was measured by drawing air over a shoot attached to the bush by means of an aspirator of the Marriott's bottle type, carbon dioxide being absorbed in approximately N/30 baryta, and estimated by subsequent titration with N/30 HCl, using phenolphthalein as indicator. The CO_2 present in the air and that given off in respiration was determined at each temperature, and subsequently, after illumination, that unabsorbed in photosynthesis. A shoot, bearing in each case leaves with an area of approximately 100 sq. cms., was enclosed in a glass vessel by means of a divided cork, and air drawn over the leaf. In the first experiments the rate of flow was 20 litres per hour, since this rate was found necessary by McLean (1920) for sugar cane, but was subsequently reduced to 10 litres per hour. No appreciable difference in carbon assimilation occurred at these two rates.

In the first instance the rate of assimilation was measured during the maximal light period on a clear, cloudless day, the temperature varying from 35°–37·5° over this period. The results are shown in Table 1, the rate of assimilation being given in mgs. CO_2 per 50 sq. cms. per hour in order to make them comparable with Lundegardh's figures (1931).

TABLE 1.

Assimilation of Atriplex vesicarium.

Full sunlight; normal CO_2. Temp. 35·5°–37·5°C. 2/12/31.

Time.	Assimilation in mgs. CO_2 per 50 cm.^{2} per hour.
10·00–10·30	3·57
11·20–11·40	2·63
11·50–12·17	2·42
2·57– 3·20	3·75
4·10– 4·30	3·62

The assimilation/time curve is saddle-shaped, and similar in this respect to those described by McLean (1920) and Thoday (1910). This depression is due to partial closure of the stomates during the midday period, as shown by

Maximov's field method. The midday closure has been fully investigated by Loftfield (1921).

The second point of interest is the low value for the assimilation rate of the saltbush compared with mesophytic plants. According to Lundegardh and others (1931) the assimilation rate under normal conditions of plants such as potatoes, beet, spinach, and vetch is about 9·00 mgs. CO_2 per 50 sq. cms. per hour. With saltbush the rate is considerably less than one-half this value. This low rate can probably be attributed to the leaf morphology and low chlorophyll content, the chlorenchyma being central and surrounding the vascular tissue. The low rate is entirely at variance with the view of Maximov (1929), that xerophytic plants have a higher assimilation rate than typical mesophytes.

The form of the assimilation/temperature curve indicates a definite heat adaptation, the optimum for assimilation of saltbush occurring between 40° and 45°C. These temperatures are considerably above the lethal temperature of the leaves of mesophytic plants which have been examined.

The assimilation rates at various temperatures in the critical region under natural conditions (full sunlight, and 0·03% by volume of CO_2) on a clear, cloudless day in December are given in Table 2.

TABLE 2.

Assimilation of Atriplex vesicarium.

Full sunlight; normal CO_2. 3/12/31.

Temperature.	Assimilation in mgs. CO_2 per 50 cm.^{2} per hour.
24°	2·60
29°	2·89
34°	3·27
37°	3·54
45°	3·82
50°	3·36
55°	2·00

It is clear from these figures that the optimum lies between 40° and 50°C., which is approximately the normal range of the sun temperatures during the active growing period of *Atriplex*.

It has been established that the coagulation point of protoplasm is altered considerably by the presence of salts—an application of a general principle of colloid-chemistry. The most active ions are chlorine and sulphate ions, and Lundegardh (1931) records that 0·3–0·4 N solutions of chlorides and sulphates raise the coagulation point of the cells of *Tradescantia zebrina* to between 74°–76°C.

It has already been shown by Wood (1924) that the leaves of *Atriplex* spp. contain from 8–30% of the dry weight as K and Na chlorides, and it is probable

that this high salt concentration (equivalent on the average to about 2N NaCl) may account for the high optimum temperature relations of photosynthesis.

CARBOHYDRATES OF THE TOMENTOSE SUCCULENT PLANTS

Spoehr (1919), in his monograph on the carbohydrates of the Cactaceae, showed that plants of this family contained considerable amounts of pentosans, and further that pentosan formation is dependent on the water content of the plant. With continued low water content the pentosan content increased considerably.

In an extension of this work, MacDougal, Richards, and Spoehr (1919) considered that the transformation of hexose-polysaccharides with low imbibition capacity to pentose-polysaccharides with high hydration capacity constituted the basis of permanent succulence.

In all the tomentose succulent plants from arid Australia that have been examined pentosans are present in the leaves. Microscopic examination with ruthenium red and neutral red showed that mucilage is present in the protoplasm of the aqueous tissue in all species of *Kochia* and *Bassia* that have been examined, and also in large amount in the dense non-vacuolate protoplasm of the basal cells of the hairs. In *Salsola Kali*, *Rhagodia* spp., and *Enchylaena* sp. the same distribution of pentosan is found. In *Atriplex* spp. some pentosan is present in the hypodermal water storage tissue and in the basal cells of the hairs, but the bulk of this carbohydrate is restricted to the thick-walled chlorenchyma cells which surround the veins. These cells have dense protoplasmic contents, are non-vacuolate, and contain a great deal of chlorophyll, much more so than those of the second layer of chlorenchyma which surrounds them centripetally. In this second ring of cells no pentosans are present, as shown by ruthenium red, neutral red, or by microchemical tests with HCl and orcinol. In the central cells, which contain the mucilage, a large number of small starch grains is also present.

Determinations of the carbohydrate content were made on the commonest and most widespread species, *Atriplex vesicarium*, *Kochia sedifolia*, and *Bassia patenticuspis*. The procedure followed closely that of Spoehr (1919).

One grm. of the dried leaves, together with a small amount of $CaCO_3$, was extracted twice with 100 c.c. of 95% alcohol to remove soluble sugars. This solution was evaporated, hydrolysed with 5% HCl, and the sugars determined by copper reduction. The residue after alcohol extraction was boiled with 1% HCl for three hours to hydrolyse polysaccharides such as starch and mucilage, and the sugars determined in this solution. An aliquot portion of this was incubated with pure yeast culture at 30°C. for 30 hours to remove hexoses; the solution was evaporated to dryness to remove alcohol produced in the fermentation and after solution made up to a definite volume of water. Then pentoses

were determined by copper reduction. In the case of *Atriplex*, which contains about 25% protein on the dry weight basis, it was necessary to remove this with basic lead acetate, and subsequently excess of this reagent by sulphuric acid. In the case of *Bassia* and *Kochia* the percentage of protein is small, and it was found that its removal introduced errors into the sugar estimation greater than if the protein were not removed. For estimation of the sugars Somogyi's (1926) modification of the Schaffer-Hartmann method was used. The results of these analyses are given in Table 3.

TABLE 3.

Carbohydrates in Percentage of Dry Weight of Arid Plants. August, 1931.

	Atriplex vesicarium.		*Kochia sedifolia.*		*Bassia patenticuspis.*
	Koonamore.	Adelaide.	Koonamore.	Adelaide.	Koonamore.
Total polysaccharides	9·37	11·13	5·58	5·46	11·61
Hexose polysaccharides	7·69	9·02	3·58	3·29	9·15
Pentosans	1·68	2·13	2·00	2·17	2·46
Soluble sugars	1·94	1·26	1·51	2·08	1·03

Analyses of plants grown near Adelaide (rainfall 25 inches per annum) and at Koonamore (8·12 inches per annum) were analyzed in August, at the end of the wet season. The pentosan content in all cases is approximately the same (about 2%), and no difference at this time was apparent owing to difference in habitat. Both places, however, had good falls of rain in the two months previously. The amounts of carbohydrates in all cases approximate closely to those of *Opuntia versicolor* given by Spoehr (1919), but the amounts of pentosan are less, generally speaking, than in the more mucilaginous species, such as *Opuntia phaeacantha.*

Spoehr showed that the pentosan content increased with the increasing aridity, and this is paralleled in the case of *Atriplex vesicarium* at least, as the following analysis in Table 4 shows.

TABLE 4.

Carbohydrates in Percentage of Dry Weight. December, 1931.

	August, 1931.	December, 1931.
Water	87·00	73·00
Total polysaccharides	11·13	20·83
Hexose polysaccharides	9·02	14·01
Pentosans	2·13	6·82
Soluble sugars	1·26	1·57

The plants for the December analysis were gathered in the middle of the dry season in Southern and Central Australia. It will be seen from the table that the polysaccharides show a considerable increase in December compared with the August analyses, and in particular that the pentosans show a large increase with increasing aridity and with decreased water content of the leaves.

Trials on the swelling relations of the leaves showed them to be comparable

to *Opuntia* spp. The greatest amount of swelling in fresh dried sections occurs in pure water; in 0·01 M KOH solution the swelling is almost as great, whilst little swelling occurs in 0·01 M citric acid solution. The imbibitional swellings parallel those of pentosan-protein mixtures, such as agar and gelatine, which have been demonstrated by MacDougal (1916). In some of the tomentose succulent plants, such as *Atriplex*, the protein content is as high as 25% of the dry weight, so there is no doubt that this bicolloid forms an efficient absorbing complex.

One gramme of the leaves of *Atriplex vesicarium* and *Kochia sedifolia* were air dried for 40 days at a constant humidity of 30% at 15°C., and introduced into a Torricellian vacuum. Under these circumstances a depression of about 1 mm. of mercury occurs in each case at 15°C. and 760 mm. pressure.

These results leave little doubt as to the relation of the tomentose succulent-leaved plants to the stem succulents with regard to their carbohydrate economy. It is obvious that the persistence of these plants in the driest habitats in arid Australia is due in large measure to the imbibition of water through their leaves, owing to the presence of a concentrated cell sap, and also of pentosan colloids, to an uncutinized epidermis, and also to the retention of this water by the colloids during wilting. In these plants at least the mechanism of Maximov's concept of the xerophyte in resisting permanent wilting is clear.

It is probable that the imbibition of water from the atmosphere, in addition to its retention, accounts for the greater drought resistance of the tomentose succulent-leaved plants when compared with the Cactaceae. The latter are found only in the wetter zones of arid regions where rainfall is not uncertain, whereas the tomentose succulents occur in most abundance between the 8 and 5 inch annual isohyets in arid Australia.

SUMMARY.

Contrary to the views expressed by Maximov, the succulent-leaved plants, such as the saltbush, do not show a higher photosynthetic activity than mesophytic plants, but the hourly rate per unit area under natural conditions of illumination and carbon dioxide content of the air is less in the shrub steppe plants than in typical mesophytes. In saltbush the assimilation/temperature curve also differs from those of mesophytic plants, the optimum rate of carbon assimilation occurring between 40° and 45°C.

Analyses of leaves have shown that in plants of arid communities the carbohydrate flux is diverted in part to pentosan metabolism along two lines: first, to mucilage (pentosan) formation in the succulent-leaved plants, and secondly, to pentose aromatic condensation products in the tissues of the leaves in the sclerophyll-leaved plants. Swelling and imbibitional relations show that it is to pentosan-protein colloids that the drought resistance of the succulent-leaved plants is due.

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STUDIES IN CENTRE OF GRAVITY CHANGES

No. 3

AN INVESTIGATION OF THE SEX DIFFERENCE IN RESPIRATION, BY MEASUREMENT OF THE RESPIRATORY VARIATION IN THE CENTRE OF GRAVITY.

by

F. S. COTTON

(From the Department of Physiology, University of Sydney).

INTRODUCTION.

Many investigators have attempted to decide whether the relative preponderance of thoracic respiration was an essential or imposed characteristic of the female sex. Most of the investigations were carried out many years ago, and in the meantime radical changes have taken place in women's clothing, which now restricts abdominal movement less than formerly. The first reference to a sexual difference in the character of the respiration discoverable was by Boerhaave in 1744. He concluded from his observations that the difference was manifested even in the case of infants. Boerhaave regarded the thoracic type of breathing as a provision of nature against the restriction of a distended uterus to diaphragmatic movement. All subsequent workers, however, except Hutchinson, agree that the difference is not manifested in children. Hutchinson (1846) concluded that a greater thoracic respiration was characteristic of the female sex, and that even in girls of 11 to 24 years of age the respiration could not be counted by observing the abdomen. On the other hand, Mays (1887), who examined 82 Indian girls from 10 to 20 years of age, found that the extent of abdominal movement in respiration did not differ from that of men. The work of Wilberforce Smith (1890), who took respiratory tracings from different regions of the thorax and abdomen in men, "women dressed and corseted in the usual manner," and "women habitually wearing no corset," and that by Kellogg (1895) supports the conclusion of Mays.

A comprehensive review of respiratory movements is given by Cripps (1924), which deals with the published work up to 1924. In this there are but two accounts touching the subject of sex difference, which are supported by quantitative data. These are by Fitz (1896) and Halls Dally (1908). The latter, who used an orthodiagraphic method, did not examine women wearing un-

restricting clothing, so that his results agreed with previous findings, that a lesser degree of diaphragmatic movement is characteristic of females. Fitz, however, made a special study of groups of men and women, including 35 of the latter who did not wear constricting clothing. He concludes that "between girls and women and boys and men there is little or no difference in respiratory type." Whether his results justify this conclusion will be discussed later.

METHODS EMPLOYED IN PRESENT INVESTIGATION.

As the application of any apparatus to thorax or abdomen disturbs the normal movements, it is desirable to obtain records by a method which avoids the use of a constricting band to thorax or abdomen. This is possible by making a series of measurements in men and women of the respiratory changes in the centre of gravity of the body in the supine and sitting postures.

In an earlier communication on changes in centre of gravity (this Journal, vol. 8, p. 53) it was pointed out that in the supine position the centre of gravity moved to and fro as a result of visceral displacement during respiration. In the supine position, movement of the thorax in a vertical direction does not affect the conditions of equilibrium. In the sitting posture, however, the thoracic movements during inspiration produce a caudal displacement of mass, which will be indicated by movement of the balance index. Diaphragmatic movements also contribute to this change, since the abdominal wall is pushed a little anteriorly when the diaphragm descends. The extent of centre of gravity change in each of these two cases may be quantitatively assessed (see previous communication) as so many grms. cms. of mass displacement, so that convenient comparisons may be made.

If we now calculate the ratio of the measurement in the supine posture to that in the sitting posture, *it is clear that any decrease in the thoracic component must increase the ratio, and vice versa,*[1] *and if these ratios for a series of men and women show a significant difference between the two sexes we may conclude that this is a sexual characteristic.*

For the purpose of further explanation of the details of the method, tracings are given in figs. 1 and 2 to show the various types of record obtained.

It will be seen that the effect of increasing the abdominal component of respiration by augmenting the diaphragmatic movement is to greatly increase the amplitude of the tracing. Tracing C shows, on the other hand, a lesser amplitude than normal, although the breathing is much deeper. This shows that the increase of thoracic movement tends to decrease relatively the height of the tracing in the supine posture.

[1] If during the inspiration there is a measurable displacement of portion of the thorax in the cranial direction, it will slightly decrease the numerator of this ratio, which would merely enhance the change in a manner consistent with the meaning of the ratio.

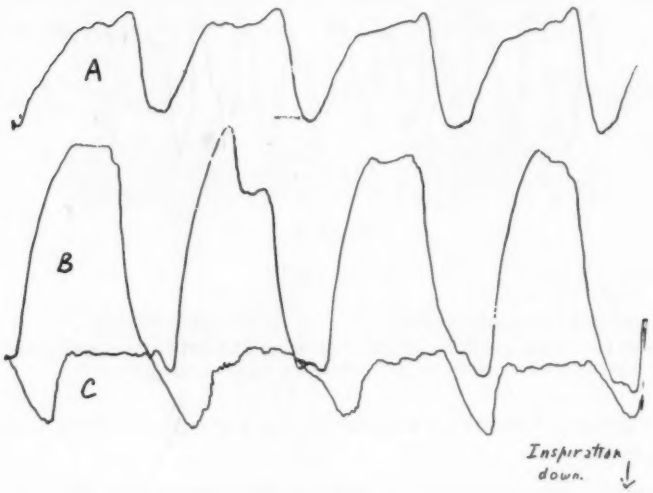

Figure 1.

Showing various types of respiratory tracings obtained by means of the centre of gravity balance table (supine posture).

A. Tracing of normal breathing. B. Tracing taken while the same subject was consciously making more ample diaphragmatic movements. C. Tracing of deep breathing, chiefly thoracic.

Before measurements of the tracings were made, however, it was considered advisable to take into account the effect of the heart-beat in order to eliminate a disturbing factor. When the subject holds the breath, as shown in fig. 2, the heart-beats are recorded separately. The amplitudes of these excursions are not the same for the supine and sitting postures, nor is the ratio of these found to be the same in different subjects. Hence the respiratory variations as obtained must be measured as gross excursions, whereas we are really concerned with the net amplitudes. Since the frequency of the heart-beat is several times that of the respiratory variations, we have the equivalent of a series of quick, small vibrations superimposed upon slow, relatively large waves. Hence it would be sufficiently accurate for our purposes to measure the gross amplitude and deduct the height of the smaller waves.

A tracing typical of that obtained by means of the balance table, showing these composite waves, is shown in fig. 2.

For convenience the ratio of the abdominal to the thoracic component of respiration, as measured in this way, will be referred to as the ratio Ab/Th.[2]

[2] It should be emphasized that this ratio does not measure the relative amounts of air inspired, due to the thoracic and abdominal components of respiration.

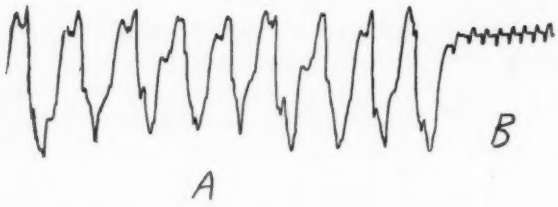

Figure 2.

Showing a tracing taken with the subject supine:

A. Breathing normally.

B. Holding the breath.

Part A of the record shows the composite tracing of heart-beats superimposed upon the respiratory waves. Part B shows the excursions due to the heart-beat only.

The necessary records for computing the ratio Ab/Th were taken in the following stages:

1. The subject, after resting some ten minutes, after ordinary laboratory activity climbs on the balance table, and assumes the supine posture. 2. The balance table is equilibrated by means of sliding weights attached to the side so as to bring the writing index about horizontal. 3. The drum is set in motion and allowed to move slowly for some time, until the respiratory excursions become reduced to a reasonably constant level from their typically greater initial extent. This usually occupied about three minutes. 4. The drum is then pushed on a little by hand to make a break in the tracing, and a further tracing taken for one or two minutes. 5. The subject is then asked to hold the breath for about ten seconds, when a tracing of the heart alone is recorded. 6. The whole procedure 2 to 6 is repeated with less initial delay after a wooden support to keep the subject still in the sitting posture[3] is placed in position on the balance table. 7. A further tracing is taken of the subject in the supine posture, so as to eliminate the effect of any progressive change that may be taking place in the mean depth of breathing.

To compute the ratio for the subject the mean net excursions (total amplitude less amplitude of the heart-beat) are reckoned from each of the two supine tracings, and the average is then divided by the mean net excursion of the sitting tracing.

In order to test whether variation in the thoracic component relatively to the abdominal component does alter the ratio of these excursions in the manner predicted, a set of tracings was taken with the same subject: (1) Under normal conditions. (2) With the abdomen constricted by means of a

[3]It is found in practice that essentially the same quantitative result is obtained with the subject sitting with the trunk inclined at about 80° to the horizontal as with the subject sitting bolt-upright. As the former position was found to be much the more comfortable, it was therefore adhered to throughout the experiments by the provision of a supporting piece inclined at that angle. In addition, two small cushions were provided for the head and hollow of the back respectively, with the result that the subject always reported that the conditions were quite comfortable.

bandage, 20 feet long and 3 inches wide, wound tightly round it. The tracings obtained are shown in fig. 3.

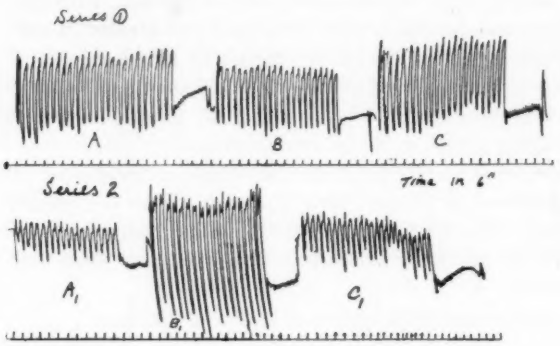

Figure 3.

A, B, and C are respectively the supine, sitting, and supine respiratory tracings taken in that sequence. A_1, B_1, and C_1 are the corresponding tracings taken with a bandage wound tightly round the abdomen of the subject.

The tracings show that the experimental reduction of the abdominal component of respiration, together with an increase in the thoracic component, has produced a marked change in the tracings in the direction indicated by the initial argument. Now the mean net excursions of A and C amount to 26·2 mm., while that of B amounts to 22·5. Hence the ratio Ab/Th for the normal conditions = 1·16. The corresponding measurements for the second series of tracings are 12·0 mm. and 46·0 mm. respectively, so that in this case the ratio Ab/Th = 0·26. Thus the increase of the thoracic component at the expense of the abdominal component of respiration has profoundly reduced the ratio in question, as predicted theoretically.

THE CORRECTION NECESSARY TO BE APPLIED TO THE RATIO Ab/Th ON ACCOUNT OR THE INERTIA OF THE SYSTEM.

Although it was not anticipated that the effect of inertia would profoundly alter the correction factors arrived at by calculation from the data of the previous paper (this Journal, vol. 10, p. 17), it was regarded as important to evaluate these factors as accurately as possible. In order to determine the effect of this inertia factor by experimental means, a rhythmical to and fro displacement of mass in the longitudinal axis of the system was produced so as to simulate the rhythm of respiration. This was accomplished by having a small table gramophone fitted up to carry a horizontal revolving arm, to which

a sliding adjustable weight was attached. The revolution rates were from about 8 to 25 per minute. A light subject was chosen, so that the added weight of the gramophone brought the total weight to about the average. When the subject was placed supine and equilibrium established, with the writing point in position, the gramophone was set in motion at a measured rate. The subject was then requested to hold the breath for about half a minute, when a tracing was taken. The revolving weight was placed at such a distance from the centre as would give tracings of the same order of magnitude as the respiratory tracings previously found with the human subject.

Tracings were secured: A. With the subject supine, and rates of rotation of the gramophone 9 to 24 per minute; and B. With the subject sitting, with rates of rotation from 10 to 23 per minute. The resulting tracings were then examined, and the height of each individual excursion measured so as to yield an accurate mean.

The results are shown in curves A and B in fig. 4.

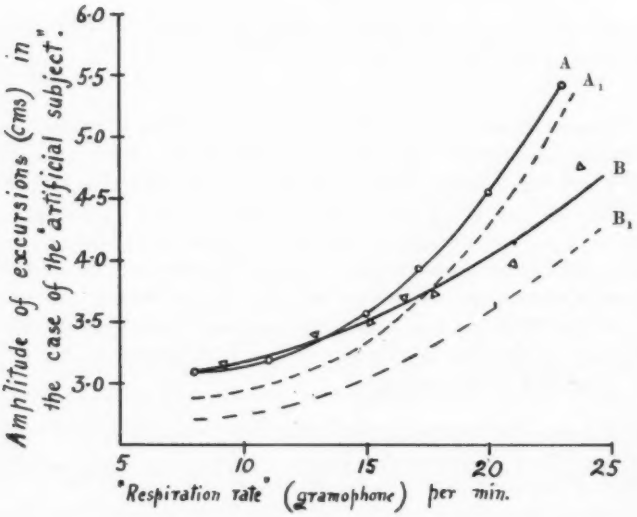

Figure 4.

Showing the calibration curves obtained by means of the "artificial subject." A for sitting posture. B for supine posture.

It will be noted that in this instance the two curves meet at a point where the inertia effect is practically negligible. This apparently conflicts with the correction factors calculated from the equations of the previous paper, where

it was shown that the amplitude of the tracing in the sitting posture may be predicted to be greater than that in the supine posture. The explanation for this is that the effects of the heart-beat have not yet been considered. When these are determined, and the net amplitudes plotted, the corrected curves A_1 and B_1 are respectively obtained. The inertia effect on the correction ratio must be determined by actual measurement of the ordinates of the two final curves A_1 and B_1. Let any ordinate of A_1 be called A, and that of B_1 be B. Then since our respiratory ratio is reckoned as Ab/Th, the correction factor must be calculated as A/B. The correction factor for any given frequency of respiration is therefore found by calculating this ratio A/B for that particular value of the abscissa.

The data for fig. 5 are obtained in this way, so that it is possible to read off from it the correction factor corresponding to any given rate of respiration.

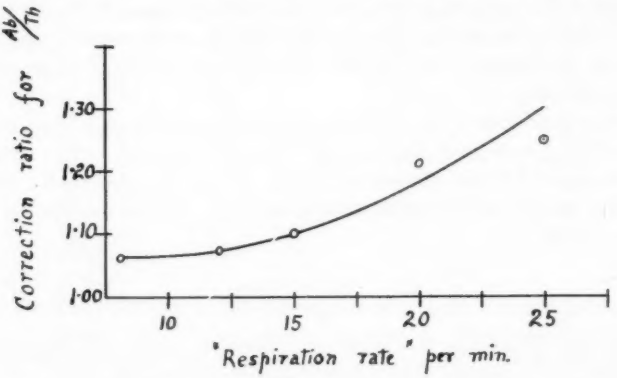

Figure 5.

Showing the effect of frequency of respiration on the correction factor for Ab/Th due to inertia.

All the individual determinations previously made were then corrected by means of this graph, so that it was now possible to make one complete group of all the men and another of all the women.

Before dealing with the final results, evidence must be offered to show that the method in its entirety is sufficiently accurate for the purpose in hand.

AN ACCOUNT OF THE TESTS MADE TO ASSESS THE COMPETENCY OF THE METHOD AS USED[4] TO YIELD CONSISTENT RESULTS.

The value of the method in attacking the present problem clearly depends upon its capacity to give concordant results on the same individual under

[4] This distinction is made, since much more accurate results could be obtained by taking longer tracings, which would, however, greatly increase the tedium of the measurements.

similar conditions of rest. In this connection it is possible for two varieties of error to affect the final result:

1. Variations due to the experimental procedure or due to some difference in the subject on different occasions. 2. Errors in obtaining the required ratio by measurement of any given tracing.

The second point will be dealt with first. Even a brief experience in respiratory tracings is sufficient to show that quite considerable variations in the amplitude of individual excursions are common, so that it is a matter of judgment, when a given tracing is taken, to determine the mean excursion by means of a reasonable number of measurements. As previously explained, it was endeavoured to obtain the tracings in question after the typical preliminary decrease had occurred. In general, some 20 to 40 excursions were recorded in each tracing. The procedure adopted was to inspect the tracing and to pick out the most uniform portion covering an extent of some 15 to 20 waves. The mean amplitude of these was then determined by measurement, and reduced to net value by deduction of the mean excursion of the heart-beat, which itself offered no difficulty.

To assess this error a set of tracings was taken and measured, and then set aside, care being exercised not to mark them in any way. The next day they were again measured, and the second series of results tabulated. The two sets of completed measurements were then compared. These are shown in parallel columns in Table 1.

TABLE 1.

Showing the Results of Duplicate Measurements made on Different Days on the same set of Unmarked Tracings.

The calculated values are the ratios Ab/Th for men.

Subject.	First Measurement.	Second Measurement.	Difference.
L.L.B.	1·76	1·80	0·04
H.M.F.	1·75	1·82	0·07
R.H.	1·52	1·53	0·01
E. McM.	1·57	1·58	0·01
H.R.K.	1·19	1·20	0·01
J.J.M.	1·41	1·27	0·14
K.V.S.	1·24	1·25	0·01
Mean	1·47	1·47	0·041

From the table it is seen that the mean error of judgment plus measurement amounts to an average of about 3 per cent.

In order to test the first point a series of duplicate tracings were obtained in the case of a series of subjects on different days. The results are set out in Table 2.

TABLE 2.

Showing the Consistency of Duplicate Measurements made upon a Group of 19 Women on Consecutive Days.

	Ratios of Ab/Th.		
No. of Subject.	First Determination.	Second Determination.	Difference.
1	1·26	1·32	0·06
2	0·94	0·99	0·05
3	1·02	1·04	0·02
4	1·16	1·34	0·18
5	1·68	1·65	—0·03
6	1·34	1·36	0·02
7	1·29	1·39	0·10
8	1·08	0·99	—0·09
9	1·56	1·70	0·14
10	0·96	1·05	0·09
11	1·44	1·21	—0·23
12	0·84	0·77	—0·07
13	1·02	0·99	—0·03
14	1·20	1·21	—0·01
15	0·98	1·31	0·33
16	1·02	1·05	0·03
17	1·49	1·21	—0·28
18	1·20	1·16	—0·04
19	1·26	1·16	—0·10
Mean	1·19	1·20	0·10

From the last column of the table it is seen that the mean absolute error (irrespective of sign) is 0·10, i.e., about 8 per cent. This means that, including all errors, it is possible to obtain a quantitative estimate of the type of respiration in any given subject correct on an average to about 8 per cent. on one examination.

It is therefore submitted that if the number of observations be made reasonably large we have in the present method a means of testing for even small differences in the quantity measured in the case of different groups of subjects.

THE RESULTS OBTAINED IN THE CASE OF GROUPS OF MEN AND OF WOMEN.

The men were all medical students, mostly between 18 and 21 years of age. The women were all university students, or teachers in training, ranging from 17 to 21 years of age, with the exception of some three or four, who were rather older. The clothing was universally of the non-constricting type.

The grouped results for both sexes are set out in Table 3.

TABLE 3.

Showing the Ratios of Ab/Th obtained in the case of Various Groups of Men and of Women.

	Men.		Women.	
Group No.	No. of Subjects.	Mean.	No. of Subjects.	Mean.
1	14	1·50	26	1·29
2	11	1·48	26	1·19
3	30	1·51	14	1·26
4	31	1·45	13	1·29
5	—	—	13	1·24
Total or mean	86	1·49	92	1·24

The table shows quite a satisfactory constancy for the group means, those for the men being apparently of an order of magnitude quite distinct from those of the women.

The values for the groups of men and of women are plotted as frequency curves A and B in fig. 6.

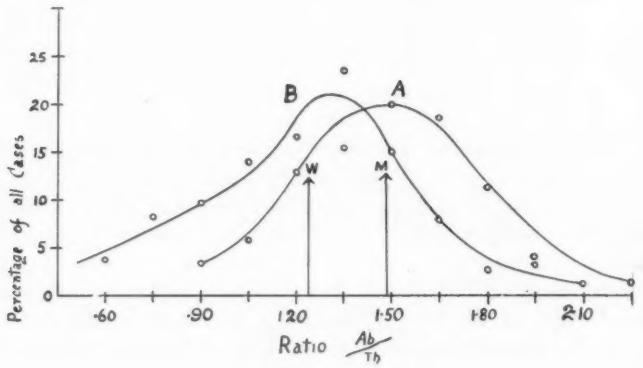

Figure 6.

Frequency curves for the complete series in each sex. A men, B women.

The lines M and W are placed respectively to mark the mean values for each series. It is evident from the curves that there is a distinct shift in the case of the women towards the lower ratio of Ab/Th, although overlapping occurs. This gives evidence of a greater thoracic component of respiration in the case of the women. The statistical data necessary to determine whether this difference is a real one, in terms of probability, are set out in Table 4.

TABLE 4.

Statistical Values for the Ratio Ab/Th for the Complete Groups of Men and of Women.

	Men.	Women.
Mean	1·49	1·24
P.E. Mean	0·021	0·021
S.D.	0·295	0·31
Coefficient of Variation ..	19·8%	24·8%
Lower Quartile	1·20	0·96
Median	1·44	1·29
Upper Quartile	1·59	1·41
P.E.	0·19	0·22
Total No. of Subjects ..	86	92

It will be seen from the table that the ratios for the men are rather more uniform than those for the women, as shown by the lower coefficient of variability, 19·8 per cent. for the men, as against 24·8 per cent. for the women. The rest of the statistical values, on the other hand, show a closer approximation. The P.E. of the mean is seen to be 0·021 in both cases, indicating that there are equal odds that these values are within 0·021 of the correct figures. This would imply that the difference between the two groups is quite definite.

To apply the orthodox statistical test for the reality of this difference between the groups, we note first that the difference between the means is 0·25. Now the P.E. of this difference is given by the expression

$$\text{P.E. (difference)} = \sqrt{(0{\cdot}021)^2 + (0{\cdot}021)^2} = 0{\cdot}029.$$

Hence the ratio of the actual difference between the means to the P.E. of that difference $= \dfrac{0{\cdot}25}{0{\cdot}029} = 7{\cdot}9$, whence from the tables of probability it may be seen that the odds against such a difference between the two groups occurring by chance are more than ten million to one.

In several instances, where previous workers had examined women wearing constricting dress, distinct differences in the tracings were reported when the dress was loosened. Although in the present series of subjects the dress was of the non-constricting type, it was considered important to determine when any such difference occurred in the tracings when the dress was thoroughly loosened. To this end some 22 of the women were examined in this way. The mean ratios for Ab/Th were found to be 1·21 before and 1·18 after complete loosening of the dress. This difference is small, and is readily shown to be in accord with the variation of sampling. Hence we conclude that the dress worn by this group of women did not affect their normal values for Ab/Th, so that the description of the group of women examined, as wearing unconstricting dress, is quantitatively confirmed.

Since the question as to whether the ratio Ab/Th is affected by physical training has an important bearing on the reality of the sex difference recorded

above, the data were examined from this point of view. The subjects of each sex were divided into a less athletic and a more athletic group. The mean ratios were then found, for the women, less athletic group 1·30 and more athletic group 1·27, and for the men, less athletic group 1·46, and more athletic group 1·49. These differences are in accord with the errors of sampling, so that the ratio in question is not a function of the degree of athletic training. This result supports the conclusion that the respiratory difference observed is a true sex difference.

In comparing these results with those quoted in the introduction it should be emphasized that *the most systematic set of quantitative data on this question that the writer can find are those of Fitz,* whose data will now be examined in detail to discover *how he arrived at the opposite conclusion.*

The method of Fitz was to take simultaneous tracings from the most freely moving regions of abdomen and thorax respectively, and by measuring the excursions of the tracings in each instance, to compute the ratio in the latter case to that of the former, as Th/Ab. But Fitz's tables show so great variation in these ratios as to suggest that they are affected by experimental error. In the case of boys the minimum ratio is 0·6 and the maximum 9·43, and in the case of the women with unconstricting clothing the minimum is 0·0 and the maximum 8·18. The other series show similar degrees of variation between the smaller and larger values obtained. Fitz did not examine his data statistically. He did not even determine the mean value for his groups. The complete data are not available for comparison between the men and the unconstricted women. In the tables, however, are shown the proportions of ratios between certain ranges for men, boys, and unconstricted women. These are given in Table 5.

TABLE 5.

Giving the Percentages of Cases of Men, Boys, and Unconstricted Women between Certain Ranges of Ratio Th/Ab.

	1	2	3
Ratio of Th/Ab ..	0·0–0·5	0·5–2·0	Over 3·0
Men	27·4	66·9	5·7
Boys	28·6	62·8	8·6
Unconstricted Women	26·5	58·7	14·8

The general distribution of values for the boys is clearly intermediate between that for the men and the unconstricted women.

Hence, if it can be proved that the difference between the groups of women and boys is real, then so also must be the difference between the groups of women and men. In order to test the matter, the statistical values were determined for the tabulated groups of boys and of women. In each case the highest value was eliminated from the group, since the deviation of these aberrant values from the mean were 10·0 and 24·8 times the respective S.D.s, whereas

even a fourfold ratio would suffice to exclude them. The rest of the data was then examined in the usual way and the contents of Table 6 computed therefrom.

TABLE 6.

Showing the Statistical Data for the Ratio Th/Ab for Fitz's Groups of Boys and Unconstricted Women.

	Ratio of Th/Ab.	
	Boys.	Unconstricted Women.
Mean	0·74	1·18
P.E. of Mean	0·04	0·08
S.D.	0·35	0·69
Coefficient of Variability ..	47%	58%
Lower Quartile	0·43	0·48
Median	0·58	0·93
Upper Quartile	0·90	1·79
P.E.	0·23	0·65
Number of Subjects	34	34

It will be evident that while there is a substantial difference between the mean ratios, 0·74 and 1·18 respectively, the high coefficients of variability, namely, 47 per cent. and 58 per cent., are a sufficient deterrent from the drawing of conclusions by mere inspection, so that a more exact examination must be instituted before any precise statement can be made. This apparently Fitz did not do, so that he missed the significance of his own results. Now the reality of this difference of the two means depends upon their respective probable errors shown in the second row of the table.

The P.E. of this difference may be computed as previously, so that it is equal to $\sqrt{(0{\cdot}04)^2 + (0{\cdot}08)^2} = 0{\cdot}089$. Hence the reality of this difference depends on the ratio $\frac{1{\cdot}18 - 0{\cdot}74}{0{\cdot}089} = 4{\cdot}5$, whence from the tables of probability the odds against such a group difference occurring by chance is rather more than 1,000 to 1. From this it is evident that the difference between the groups of men and unconstricted women is even more certainly a natural sex difference. Hence we may conclude that the only other available quantitative data on this subject, which had been adduced to prove the opposite of the conclusion arrived at in this paper, in reality helps to confirm it.

SUMMARY.

1. A brief comparison is made of the work of previous investigators on this problem.

2. An account is given of the manner in which the measurement of the ratio of centre of gravity displacements in the supine and sitting postures may

be used to distinguish relative changes in the thoracic and abdominal components of respiration.

3. Duplicate measurements made on a score of subjects show a mean error of about 8 per cent. in the determination of the ratio Ab/Th by this method, *as used*. By increasing the length of tracings this error may be much reduced.

4. The mean value of this ratio was found to be $1{\cdot}49 \pm 0{\cdot}02$ in the case of a group of 86 men, with a S.D. of 0·295, and a coefficient of variability of 19·8 per cent.

5. The corresponding values for a group of 92 women wearing unconstricting clothing were: Mean $1{\cdot}24 \pm 0{\cdot}02$, S.D. 0·31, and coefficient of variability 24·8 per cent.

6. When these data were examined statistically it was shown that this difference of 0·25 between the grouped results for men and for women is a real one, with a probability of several millions to one.

7. Athletic and non-athletic subjects were found to exhibit, on an average, the same ratio characteristic for their sex, within the error of sampling.

8. A special test made on 22 of the women subjects (unselected) showed that no measurable difference in the mean value of the ratio Ab/Th was produced by a thorough loosening of the dress, indicating that the clothing originally exercised no appreciable restraint on diaphragmatic movement.

9. A detailed examination is made of the data of Fitz, who has published the only quantitative data that the writer can find, from which the opposite conclusion from the present communication was drawn.

10. It is shown from the internal evidence of Fitz's data that he drew the wrong inference from his results, and that his work confirms the present results with a probability of more than 1,000 to 1.

In conclusion, I wish to express my thanks to Professor H. Whitridge Davies and to Associate Professor Henry Priestley for encouragement and criticism, and to Professor A. Mackie, Miss E. Redfern, and Miss E. M. Hindmarsh for their assistance in connexion with women subjects.

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[5] Quoted from Cripps.

THE INFLUENCE OF THYROXIN ADMINISTRATION AND OF THYROIDECTOMY ON THE RESPIRATION OF ISOLATED MUSCLE TISSUE

by

C. STANTON HICKS

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

(Submitted for publication 18th April, 1932.)

Hyper-activity of the thyroid gland has long been known to be associated with increased oxygen consumption. and Magnus Levy (1896) believed that the thyroid secretion could influence the tissues by way of the nervous system. The clinical association of increased muscular tonus and activity has led to the belief that this may be the cause of increased metabolism, but Aub, Bright and Uridil (1922) have shown that urethanized cats under treatment with thyroxin can have a raised metabolic rate of +13%, although the muscles are flaccid. Abderhalden and Wertheimer (1927) suggest that the effect of thyroxin on metabolism is by way of the sympathetic nerves. Experiments are not wanting, however, which point to the specific thyroxin action being entirely peripheral, and some of these are worthy of mention. Adler (1920) showed that hibernating hedgehodgs, either with heat-regulating centre removed, or after treatment with ergotoxin, could be wakened from their winter sleep with the associated rise of body temperature and heightened metabolic rate, in the same manner as normal hedgehogs. He concluded that thyroxin acts on the tissues directly.

Mansfeld and Pap (1920) attempted to increase or diminish the amount of tissue oxidizing hormone (catalyst) by cooling or warming rabbits prior to killing. From measurements of the glucose usage of the perfused surviving heart, they concluded that cooling increased tissue oxidations. Thyroidectomy prevented this increase, and the inference is that thyroxin acts on the tissues directly. Mansfeld had already shown (1913) that the heart removed from a hyperthermic animal utilized more glucose from the perfusion fluid than a normal heart, and O. Loewi (1914) has confirmed this result, which points to a hormonal influence upon the cardiac muscle oxidations.

With the isolation of thyroxin by Kendall, Plummer and Boothby (1921) were able to conduct experiments on myxoedema patients, which indicated that

thyroxin might be acting as an oxidative catalyst, but did not actually prove this to be the case. Cori (1921) found that the temperature of a human cretin when exposed to cold fell more rapidly than a normal subject, and was able to show later (1923) that rabbits deprived of their thyroid glands, when cooled for ten minutes in a water bath at 25°C., not only showed a very much greater fall of body temperature than the control animals, but a greatly diminished rate of recovery. Boothby and Sandiford (1924), and also Boothby and Kendall (1924), by experimental determination of the rate of "decay" of thyroxin in a case of myxoedema (a rate which followed an exponential curve) were led still further to the conception of thyroxin as an oxidative catalyst. Rohrer (1924) showed for the first time that administration of thyroid gland substance to rats led to an increased oxygen uptake of the isolated and surviving tissues of liver, kidney, and muscle, while Ahlgren (1924), using Thunberg's methylene-blue technique, found that muscle from thyroidectomized rabbits had a lower rate of oxygen consumption *in vitro* than that of normal rabbit muscle. He also found that addition of traces of thyroxin to the surviving tissues was followed by an increase in the rate of reduction of methylene-blue. The experimental evidence in this latter case seems conclusive enough, but whether the technique measures the normal *in vivo* effect of thyroxin in this case is doubtful. Holden and I made similar experiments at Cambridge in 1924, but could discover no effect of thyroxin on oxygen uptake of minced rabbit muscle, measured in a Barcroft manometer, and the known delay in metabolic action seen *in vivo* after thyroxin administration would suggest caution in interpreting Ahlgren's results.

Dye and Maughan (1929) demonstrated that muscle tissue from thyroidectomized pups had an oxygen uptake some 20% below that of normal muscle tissue, and further showed that washed muscle tissue under similar conditions had a succinoxidase activity of 40% below that of normal washed muscle. Anselmino, Eichler, and Schlossmann, however (1929), found that previous injection of thyroxin did not increase tissue respiration *in vitro*. They used liver, spleen, and kidney, and found in the latter instance increased anaerobic glycolysis. They conclude that thyroxin affects principally the anaerobic phase of metabolism.

We now report results of experiments on the respiration of isolated muscle from rats, carried out from time to time since 1928. The results in Table 1 were obtained with the muscle of (1) animals thyroidectomized at three months of age, and utilized for experimental purposes at eight months; (2) normal rats of similar age; and (3) others which had been injected with 0·7 mg. thyroxin "Roche" weekly, for six weeks previously. These last animals lost on the average 10% of their body weight, and were in a poor condition. The figures in the table and the graph illustrate the rates of oxygen uptake of surviving muscle tissue in oxygen at 37°C., the values being expressed in cubic millimetres of oxygen at N.T.P. per gramme of tissue. The buffer solution consisted of N/15 di-sodium

phosphate and 0·9% sodium chloride. Mixed leg muscle was removed from the animal immediately after being killed by a blow on the neck. It was rapidly minced at 0° to a fine "mush," and then weighed rapidly in 0·5 grm. portions, which were transferred to 2·5 c.c. of buffer solution in Barcroft differential manometer flasks, and thoroughly mixed by agitation.

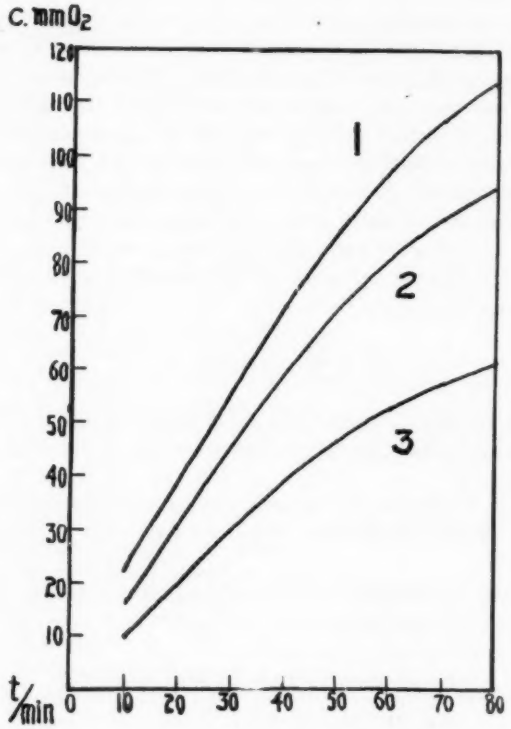

Figure 1. Oxygen-uptake per gramme muscle tissue of (1) rats previously injected with thyroxin, (2) control rats, (3) rats thyroidectomized five months previously.

Observations on four specimens of mixed muscle tissue from each animal were made simultaneously, results being discarded when a single estimation departed more than 10% from the mean of the four.

In this way results have been obtained which show that the amount of thyroxin in the tissues prior to death determines the rate of oxygen uptake of the surviving tissues.

DISCUSSION.

In comparing these results with those of Anselmino and his colleagues, it must be noted that the protocols here presented are those selected from a total of fifteen thyroxin injected animals, the remainder being rejected for the reasons previously given. Anselmino's paper gives a very critical examination of his own experiments, in which he used the Warburg technique, and it is clear that under his experimental conditions, where rats received doses of thyroxin "Roche" from 1 mg. to 8 mgs., only kidney tissue showed an increased O_2 uptake, this being equal to three times the value of the mean error of the method. He did not use muscle tissue, and it does not appear that his animals were injected for so long a period as our own. In his experiments the increase of oxygen consumption of the rats was approximately 15%. In our cases, although the metabolic rate was not measured, the animals lost an average of 10% of body weight, and were in extremely poor condition when killed, owing to hyperthyroidism. It is concluded from this that the animals in the present instance were much more under the influence of thyroxin than were those of Anselmino and his co-workers.

SUMMARY.

The oxygen uptake of finely-minced rat muscle tissue at 37°C. is increased by 20% when the animal receives hypodermic injections of 0·7 mg. thyroxin weekly for six weeks.

Muscle tissue from rats five months after thyroidectomy showed a diminution of oxygen uptake of 33%, as compared with that of tissue from control animals.

The suggestion of Maughan and Dye, that thyroxin acts by catalyzing oxidation in the tissues, is thus supported.

Respiration by Isolated Muscle at 37°C.

c.mm. O_2 Absorbed per gramme of Tissue.

(1) *Thyroidectomized Rats.*

	10 mm.	20 mm.	30 mm.	40 mm.	50 mm.	60 mm.	70 mm.	80 mm.
	10·2	20·1	30·3	40·0	46·7	53·1	58·6	60·0
	11·4	21·1	29·4	41·2	45·9	50·2	56·6	56·0
	9·9	18·2	27·5	32·5	41·2	49·0	53·1	58·3
	10·2	22·0	29·1	38·9	46·1	54·0	57·7	61·1
	11·6	23·0	31·1	42·0	45·9	53·3	59·3	60·2
Average	10·6	20·8	29·5	38·9	45·1	57·9	57·0	59·1

(2) *Normal Rats.*

	10 mm.	20 mm.	30 mm.	40 mm.	50 mm.	60 mm.	70 mm.	80 mm.
	16·0	30·0	45·9	60·1	72·7	82·3	88·1	91·0
	18·1	31·1	46·3	61·2	70·1	83·4	87·1	92·1
	15·5	30·2	45·6	59·2	71·1	82·1	88·1	91·3
	15·3	30·1	45·4	59·9	71·3	82·2	88·8	90·0
	16·0	33·1	48·8	62·3	74·0	84·1	88·0	89·1
	14·7	30·0	45·7	60·6	73·0	81·1	88·0	91·0
Average	15·9	30·7	46·3	60·5	72·2	82·0	88·1	90·7

(3) *Rats previously injected with Thyroxin.*

	10 mm.	20 mm.	30 mm.	40 mm.	50 mm.	60 mm.	70 mm.	80 mm.
	25·1	40·0	55·5	73·7	83·4	91·9	104·8	104·4
	24·4	38·3	56·4	71·6	84·4	92·2	100·3	100·7
	23·2	38·1	56·0	72·2	86·6	98·5	106·2	114·0
	23·0	38·5	57·1	78·0	89·9	99·0	109·1	120·0
	22·5	37·7	57·0	74·4	84·4	98·9	107·0	116·6
	23·1	39·0	54·0	74·4	87·1	99·9	109·1	119·9
Average	23·5	38·6	56·5	72·3	85·9	96·7	106·1	112·6

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